REMARKS

This Amendment is responsive to the Office Action mailed April 29, 2009. With this submission, claim 1 has been amended. Claims 1-13 are pending; claims 1-7 are under consideration; and claims 8-13 are withdrawn.

Support for the instant amendment can be found throughout the specification as filed, e.g., at page 1, first paragraph; and page 4, line 8 through page 5, line 13. No new matter has been added. Reconsideration and withdrawal of the rejections made in the above-referenced Office Action are respectfully requested in view of the following remarks.

Claim Objections

The Office Action objects to claim 1 for alleged lack of correspondence between the preamble and the method steps recited in the body of the claim.

In response, and without acquiescing to the propriety of the objection, Applicants submit that the instant amendment is responsive to the present objection, and respectfully request withdrawal of the same.

Claim Rejections – 35 U.S.C. § 112, Second Paragraph

The Office Action rejects claim 3 under 35 U.S.C. § 112, second paragraph, as allegedly indefinite for recitation of an "N-terminal fragment" of a p63, p44, or p40 isoform of Cochlin and for recitation of a 16kDa "N-terminal fragment" of Cochlin. In particular, the Office Action asserts that the terminology "N-terminal fragment" renders the scope of the claim unclear because an "N-terminal fragment" might refer to the N-terminal amino acid of a protein, to the N-terminal half of the protein, or to various N-terminal portions thereof. The Office Action also

asserts that the claim is unclear because "no specific or limiting definition for this term has been provided in the instant specification" (Office Action at page 3, Section 9).

In response, Applicants submit that the claim 3 is clear and definite. Applicants further submit that the Office has the initial burden to show that a claim is not clear and definite, and the Office has not met its burden. In particular, Applicants submit that they are not required to provide a "specific or limiting definition" to render each and every claim term clear and definite. Indeed, Applicants are not required to provide a "specific or limiting definition" for even a single claim term. Moreover, Applicants submit that one of ordinary skill in the art would immediately know what is encompassed by "an N-terminal fragment of a p63, p44, or p40 isoform of Cochlin, or a 16kDa N-terminal fragment of Cochlin," even when no "specific or limiting" definition for an "N-terminal fragment" has been provided in the specification. Accordingly, Applicants respectfully request withdrawal of the rejection under 35 U.S.C. § 112, second paragraph.

Claim Rejections – 35 U.S.C. § 103(a)

The Office Action maintains the rejection of claims 1-4 under 35 U.S.C. § 103(a), as allegedly unpatentable over Ikezono et al. (*Biochimica et Biophysica Acta* **1535**:258-65, 2001; hereinafter "IKEZONO") in view of The Dictionary of Medicine, definition for the term "perilymph" (2000); Peter Collin Publishing, London; Retrieved October 21, 2008 from http://www.credoreference.com/entry/1051726/), Magal et al. (U.S. Patent 6,274,554 B1; hereinafter "MAGAL"), Wall et al. (*Otolaryngol. Head Neck Surg.* **112**(1):145-53, 1995; hereinafter "WALL"), and Botstein et al. (U.S. Patent No. 6,913,919 B2; hereinafter "BOTSTEIN"). In particular, the Office Action asserts that IKEZONO discloses a method for

detecting the protein product of the *Coch* gene in homogenized inner ear tissue samples. However, the Office concedes that IKEZONO fails to specifically teach the detection of Cochlin in a body fluid existing in the middle ear. For this missing feature, the Office relies on the Dictionary of Medicine, MAGAL, WALL, and BOTSTEIN.

In addition, the Office Action states that Applicants' arguments have been considered but not found persuasive. With regard to the specificity of Cochlin expression, the Office Action asserts that one of ordinary skill in the art would understand that Cochlin expression is specific to the inner ear (as compared to serum, for example) based upon the cited art. The Office further notes that the claims do not require positive or definite determination of perilymph fistula based on Cochlin detection alone, but rather 'using the detected existence of Cochlin **as an indicator of the possibility of a perilymph fistula**' (Office Action at page 11, third paragraph). The Office also alleges that the perilymph is a part of the inner ear as evidenced by the Dictionary of Medicine, and therefore BOTSTEIN's reference to the "inner ear (cochlea)" does not exclude perilymph.

With further regard to the specificity of Cochlin expression and localization, the Office disagrees with Applicants' assertion that MAGAL appears to disclose that proteins are capable of penetrating into the perilymph *when accompanied by a suitable vehicle and/or agent.* Instead, the Office suggests that one of ordinary skill in the art would understand MAGAL to teach that 'proteins do penetrate the membrane of the round window into the perilymph of the inner ear' generally (Office action at page 12, lines 11-13). The Office also alleges that the role of the microspheres and liposomes disclosed by MAGAL is to "allow for the gradual release of protein and not for penetration into the perilymph" (Office Action at page 12, lines 13-16).

The Office was also not persuaded by Applicants' argument with respect to the rationale set forth in the rejection of the claims under 35 U.S.C. § 103(a). In particular, Applicants submitted that it is not possible to argue that Cochlin expression is specific to perilymph AND that one of ordinary skill in the art would expect to find Cochlin in the perilymph as a result of leakage from surrounding tissue. In reply, the Office Action asserts that the prior art need not provide definitive data to show that Cochlin is found only in perilymph and is completely absent in other body fluids or tissues. The Office further asserts that one would reasonably expect success in using this protein as an indicator of the possibility of the presence of perilymph, even if one might not expect Cochlin to be exclusive to perilymph.

In response, Applicants submit that the claimed invention is not unpatentable over IKEZONO in view of the Dictionary of Medicine, MAGAL, WALL, and BOTSTEIN. In particular, Applicants submit that the Office has not met its burden with regard to establishing a *prima facie* case of obviousness. In order to meet its burden in this regard, the Office must establish that (1) there is some suggestion or motivation, either in the documents themselves or in the knowledge generally available to one of ordinary skill in the art, to modify or to combine the cited documents, (2) the combination of teachings must disclose or suggest all the claim limitations, and (3) there must be a reasonable expectation of success (MPEP 2142). The Office has not met its burden on all three points.

Initially, Applicants submit that the Office has failed to establish a *prima facie* case of obviousness at least because there is no suggestion or motivation, either in the documents themselves or in the knowledge generally available to one of ordinary skill in the art, to modify or to combine the cited documents as the Office asserts. IKEZONO, the primary document upon which the Office relies, discloses a method for detecting the protein product of the *Coch* gene in

homogenized cochlear and vestibular membranous labyrinthine tissues of the inner ear. However, IKEZONO fails to teach "[a] method for detecting a perilymph fistula, which comprises detecting the existence of Cochlin in body fluid existing in the middle ear, and using the detected existence of Cochlin as an indicator of a perilymph fistula." In particular, IKEZONO fails to teach detecting the existence of Cochlin in *body fluid* existing in the *middle ear*. IKEZONO also fails to teach using the detected existence of Cochlin as an indicator of a perilymph fistula.

To compensate for the deficiencies of IKEZONO, the Office relies *inter alia* upon WALL. WALL teaches that a perilymph fistula is "an abnormal communication between the inner and middle ear cavities" (WALL at page 145, first column, second paragraph). According to the Office, WALL further teaches that diagnostic methods are emerging for the detection of perilymph fistula based on detection of a marker that has passed from the inner ear (where perilymph is normally found) to the middle ear (Office Action of October 30, 2008 at page 8, second paragraph). The Office further concedes that, according to WALL, "[s]uch perilymph markers...are *unique to perilymph or cerebrospinal fluid but absent in serum* (Office Action of October 30, 2008 at page 8, second paragraph; emphasis added). WALL further discloses β2-transferrin as one such exemplary substance (WALL at page 149, paragraph bridging first and second columns). Importantly, the Office then re-interprets WALL's disclosure to assert that "proteins which are unique to perilymph can be used to detect leakage of perilymph into the middle ear" (Office Action of October 30, 2008 at page 8, second paragraph). The Office then proceeds to rely on BOTSTEIN to show that Cochlin is specifically expressed in the inner ear; the Dictionary of Medicine's definition of "perilymph" to show that perilymph is present in the

inner ear; and MAGAL to show that one of ordinary skill in the art would reasonably expect to find Cochlin in the perilymph of the inner ear, including in the tissue samples of IKEZONO.

However, the Office fails to set forth any suggestion or motivation as to *why* one of ordinary skill in the art would have turned to the teachings of BOTSTEIN, the Dictionary of Medicine, MAGAL, or even IKEZONO for a protein other than β2-transferrin. In particular, the Office has failed to set forth why one of ordinary skill in the art would specifically replace the β2-transferrin of WALL with the Cochlin of IKEZONO or BOTSTEIN, especially in view of the teachings of IKEZONO and BOTSTEIN which are lacking any disclosure that Cochlin is expressed in perilymph (or cerebrospinal fluid for that matter), *but not in serum.* Applicants further submit that neither WALL nor any of the other cited documents teach that Cochlin is unique to perilymph or cerebrospinal fluid, and absent from serum. Therefore, the Office's rejection of the claims is lacking in any suggestion or motivation for combining the cited documents.

Moreover, even assuming, *arguendo*, that one of ordinary skill in the art were to rely on WALL, BOTSTEIN, MAGAL and the Dictionary of Medicine to compensate for the deficiencies of IKEZONO, it is not enough for the Office to assert that Cochlin is specifically expressed in the *inner ear* to satisfy the Office's burden of showing that Cochlin expression is *specific to perilymph, and not present in serum.* Neither is it is not enough for the Office to rely upon such an assertion to conclude that Cochlin may be detected in a body fluid of the middle ear *such that a perilymph fistula is indicated.*

BOTSTEIN appears to disclose that Cochlin is specifically expressed in the inner ear based on work published by ROBERTSON et al. (*Genomics* **23**(1):42-50, 1994; and *Genomics* **46**(3):345-54, 1997; see attached). ROBERTSON et al. examined Cochlin nucleic acid

expression in brain, cochlea, eye, spleen, liver, kidney, lung, skin, thymus, adrenal glad, small intestine, large intestine, and sternal cartridge (see Robertson et al. at page 48, Figure 3 of *Genomics* **23**:42-50, 1994). ROBERTSON et al. detected Cochlin gene expression in brain, inner ear, and eye. *Id.* ROBERTSTON et al. failed to test serum, saliva, and cerebrospinal fluid which may be present in a healthy or diseased state middle ear. ROBERTSON et al. also failed to test perilymph. These samples are important to establish a definite method for the diagnosis of a perilymph fistula. In contrast, the instant specification discloses that Cochlin is expressed specifically and exclusively in the perilymph, i.e., not in cerebrospinal fluid, saliva, or serum, and that Cochlin protein can be considered to be a specific biochemical marker for perilymph fistula (see, e.g., page 39, lines 15-16 and Figure 2). Yet BOTSTEIN does not contain such a teaching. Neither do any of the other cited documents. Thus, the Office may not rely on BOTSTEIN's disclosure that Cochlin is specifically expressed in the inner ear (cochlea) and MAGAL's alleged showing that the skilled artisan would reasonably expect to find Cochlin in the perilymph of the inner ear as a suggestion or motivation to combine the cited art. Neither may the Office rely on BOTSTEIN or any of the other cited documents for the proposition that Cochlin expression is both unique to perilymph AND absent from serum.

Applicants emphasize that ROBERTSON et al. failed to examine *perilymph* for Cochlin gene expression. Indeed, even though ROBERTSON et al. examined cochlear tissue, the perilymph in the samples should have been washed out in the process of extracting the RNA from the tissue for mRNA expression analysis. Applicants further submit that perilymph is devoid of cells, and thus even if ROBERTSON et al. had performed Cochlin mRNA expression analysis on perilymph, ROBERTSON et al. would have failed to find any Cochlin mRNA due to the absence of cells in the perilymph (mRNA is synthesized within a cell and not secreted).

Applicants understood this to be the case and, importantly, focused their work on Cochlin *protein* (see, e.g., Figure 2). Thus, the claimed invention is even further distinguished from the cited art and from ROBERTSON, and the Office's reliance on the BOTSTEIN and MAGAL documents with regard to the specificity and localization of Cochlin expression is misplaced for these reasons as well.

Applicants further submit that even after *KSR International Co. v. Teleflex Inc.,* 550 U.S. 398, 127 S.Ct. 1727 (2007), in order to establish a *prima facie* case of obviousness, there must be some reason that would prompt a person of ordinary skill, either in the references themselves or in the knowledge generally available to skilled artisan, to modify the reference. While the *KSR* court rejected a rigid application of the teaching, suggestion, or motivation ("TSM") test in an obviousness inquiry, the Supreme Court acknowledged the importance of identifying "a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does" in an obviousness determination. *Takeda Chemical Industries, Ltd. v. Alphapharm Pty., Ltd.*, 492 F.3d 1350, 1356-1357 (Fed. Cir. 2007) (quoting *KSR International Co. v. Teleflex Inc.,* 550 U.S. 398, 418, 127 S.Ct. 1727, 1731 (2007)). Based at least on the foregoing, Applicants submit that the Office has failed to provide some suggestion or motivation for combining the cited documents.

Applicants also submit that the Office has failed to establish a *prima facie* case of obviousness at least because the cited documents fail to teach or suggest each and every element of the claimed invention. In other words, the combination of documents does not yield the invention as claimed. For example, Applicants submit that IKEZONO discloses a method for detecting the protein product of the *Coch* gene in *homogenized cochlear and vestibular membranous labyrinthine tissues* of the *inner* ear, not *body fluid* of the *middle* ear. To

compensate for the deficiencies of IKEZONO, the Office relies upon the Dictionary of Medicine's definition for the term "perilymph," MAGAL, WALL, and BOTSTEIN. However, the Dictionary of Medicine, MAGAL, BOTSTEIN, and WALL fail to compensate for the deficiencies of IKEZONO, at least because these documents, either alone or in combination, fail to teach or fairly suggest "[a] method for detecting a perilymph fistula, which comprises detecting the existence of Cochlin in body fluid existing in the middle ear, and using the detected existence of Cochlin as an indicator of a perilymph fistula." In particular, Applicants submit that the cited documents fail to teach or fairly suggest detecting the existence of *Cochlin* in *body fluid* existing in the *middle* ear. The documents also fail to teach or fairly suggest such detection *such that a perilymph fistula is indicated.*

In particular, WALL fails to compensate for the deficiencies of IKEZONO for the reasons set forth above. BOTSTEIN also fails to compensate for the deficiences of IKEZONO at least because BOTSTEIN fails to teach or fairly suggest the detection of Cochlin in a *body fluid* of the *middle* ear. MAGAL does not compensate for the deficiencies of IKEZONO either. MAGAL teaches treatment of *inner* ear disease via topical application of a formulation comprising neurturin protein, as well as a suitable vehicle for introducing neurturin into the *inner* ear. However, MAGAL fails to teach or fairly suggest the detection of Cochlin in a *body fluid* of the *middle* ear. The Dictionary of Medicine's definition of "perilymph," also fails to compensate for the deficiencies of IKEZONO at least because it fails to teach or fairly suggest the detection of Cochlin in a *body fluid* of the *middle* ear.

Applicants further submit that no *combination* of the cited documents compensates for the deficiencies of IKEZONO. In particular, Applicants submit that individually none of the documents teach or fairly suggest the detection of Cochlin in a *body fluid* of the *middle ear*. The

documents in combination also fail to teach or fairly suggest such detection *such that a perilymph fistula is indicated.* In the absence of such teachings in any one of the secondary documents relied upon by the Office, Applicants submit that the Office has failed to set forth a reasonable rationale as to why the documents in combination compensate for the deficiencies of the IKEZONO document. Accordingly, the Office has not met its burden of establishing a *prima facie* case of obviousness.

Applicants further submit that the Office has failed to establish a *prima facie* case of obviousness because the Office has failed to show that there would be a reasonable expectation of success when combining the cited documents. First, as discussed above, the Office has failed to set forth how the combination of teachings discloses or suggests each and every element of the claimed invention. Absent presentation of how the combined teachings would disclose each and every element of the claim, the Office cannot then hold that the combined disclosures of the cited art would result in a reasonable expectation of success. That is because such "success" would be based on an invention different from that claimed, and therefore not germane to the rejection of the claims as obvious. Accordingly, in view of the deficiencies described above, including the failure of the Office to show how the combined teachings would disclose each and every element of the claim, the Office has also failed to establish how the artisan of ordinary skill would have had a reasonable expectation of successfully performing the claimed method using the combined teachings of IKEZONO, WALL, MAGAL, BOTSTEIN, and the Dictionary of Medicine.

Applicants further submit that the claimed invention is not unpatentable over the cited documents insofar as the cited art teaches *away* from the instant invention. For example, at page 149, first column, first full paragraph, WALL discloses that "distinguishing perilymph from other fluids or mixtures of fluids on the basis of quantitative differences in concentration of a

marker in microliter specimen volumes will be difficult." At page 147, Table 1, WALL discloses that even the potential use of β2-transferrin, WALL's "most promising candidate endogenous marker" for identification of perilymph fistula, has its drawbacks, including the time it takes to perform the identification procedure (see also, WALL at page 149, paragraph bridging first and second columns, last sentence).

Other documents teach away from the use of β2-transferrin as well. For example, Rauch et al. (*The Laryngoscope* **110**:545-552, 2000, provided in the IDS submitted August 15, 2005; hereinafter RAUCH) disclosed years after WALL was published that "[t]he present study and other recent reports seem to indicate that the sensitivity of the [β2-transferrin] assay is unacceptably low for detection of perilymph" (page 551, first column, second full paragraph). Indeed, RAUCH also teaches away from the use of β2-transferrin insofar as RAUCH discloses that "[t]he assay technique used in the present study takes at least 18 hours to generate a results" and that as a result of such drawbacks the "β2-transferrin assay cannot provide an intraoperative answer to the perilymphatic fistula question." *Id.* Additionally, in a recent publication Ikezono et al. (*Audiol. Neurotol.* **14**:338-344, 2009; see attached) teach that "[a]lthough beta2-transferrin was thought to be a marker, a more recent study showed that, because of the relative amount of serum and perilymph in a mixed sample, electrophoretic separation of the transferrin variant might not be diagnostic [Rauch, 2000]. To date, there is no clinically relevant biochemical marker for perilymph leakage" (page 339, first column, second full paragraph).

Based at least on the foregoing, Applicants submit that IKEZONO in view of the Dictionary of Medicine, MAGAL, WALL and/or BOTSTEIN, either alone or in combination, do not disclose or suggest the claimed invention. Accordingly, Applicants respectfully request reconsideration of the rejection under 35 U.S.C. § 103(a) and withdrawal of the same.

The Office Action also rejects claims 4-7 under 35 U.S.C. § 103(a), as allegedly unpatentable over IKEZONO, in view of the Dictionary of Medicine, MAGAL, WALL and BOTSTEIN as applied to claim 1 above, and further in view of Robertson et al. (*Human Molecular Genetics* **10**:2493-2500, 2001; hereinafter ROBERTSON), the Academic Press Dictionary of Science and Technology (definition for the term "polyclonal"; Oxford: Elsevier Science & Technology (1996); retrieved October 22, 2008 from http://www.credoreference.com/entry/3144515/) and Wolfe, S.L. (Molecular and Cellular Biology, 1993, pages 790-93; hereinafter WOLFE).

In response, Applicants submit that the claimed invention is not unpatentable over IKEZONO, in view of the Dictionary of Medicine, MAGAL, WALL and BOTSTEIN as applied to claim 1 above, and further in view ROBERTSON, the Academic Press Dictionary of Science and Technology and WOLFE. In particular, Applicants refer to the comments set forth above with respect to the IKEZONO, the Dictionary of Medicine, MAGAL, WALL and BOTSTEIN documents. In addition, Applicants submit that none of the additionally cited documents in the rejection of claims 4-7 under 35 U.S.C. § 103(a) appears to compensate for the deficiencies present in the rejection of claim 1 over IKEZONO, in view of the Dictionary of Medicine, MAGAL, WALL and BOTSTEIN.

For at least the foregoing reasons, Applicants submit that IKEZONO, in view of he Dictionary of Medicine, MAGAL, WALL and BOTSTEIN as applied to claim 1 above, and further in view of ROBERTSON, the Academic Press Dictionary of Science and Technology and WOLFE, either alone or in combination, do not disclose or suggest the instant invention, and respectfully request withdrawal of the rejections under 35 U.S.C. § 103(a).

CONCLUSION

In view of the foregoing, the Examiner is respectfully requested to reconsider and withdraw the rejections of record, and allow all the pending claims.

No additional fee is believed due at this time. If, however, any additional fee is necessary to ensure consideration of the submitted materials, the Patent and Trademark Office is hereby authorized to charge the same to Deposit Account No. 19-0089.

Should there be any questions, the Examiner is invited to contact the undersigned at the below listed telephone number.

Respectfully Submitted,
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Cochlin-Tomoprotein: A Novel Perilymph-Specific Protein and a Potential Marker for the Diagnosis of Perilymphatic Fistula

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Key Words

COCH gene · Cochlin isoform · Cochlin tomoprotein · Hearing loss · Vertigo

Abstract

Background: Perilymphatic fistula (PLF) is an abnormal connection between the inner and middle ear. A procedure for obtaining definite proof of a PLF remains elusive, and methods of diagnosis remain controversial. To date, there is no clinically relevant biochemical marker for perilymph leakage. Using proteomic analysis of inner ear proteins, we have previously found unique properties of cochlin, encoded by the *COCH* gene. We detected 3 cochlin isoforms (p63s, p44s and p40s) in the inner ear tissue and a short 16-kDa isoform of cochlin-tomoprotein (CTP) in the perilymph. Since cochlin was found to be highly specific to the inner ear, we speculated that CTP might also be specific to the perilymph. The aim of this study was to determine whether CTP, a novel perilymph-specific protein, could be used as a marker for the diagnosis of PLF. ***Methods:*** By Western blotting, we investigated the specificity of CTP expression in a range of body fluids that included perilymph, serum, saliva and cerebrospinal fluid. To elucidate the detection limit of CTP, serially diluted recombinant human (rh)CTP as well as human perilymph was tested. ***Results:*** CTP was selectively expressed in all 20 perilymph samples tested, but not in 77 samples of the other body fluids. The detection limit of rhCTP was 0.27 ng or 0.022 μl of perilymph per well on Western blot analysis. ***Conclusion:*** The results strongly suggest that CTP can be a specific marker of perilymph leakage. Moreover, CTP has the potential to be a biochemical marker that allows a definitive diagnosis of the etiology of PLF-related hearing loss and vestibular disorders.

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Introduction

Perilymphatic fistula (PLF) is defined as abnormal connections between the fluid (perilymph)-filled space of the inner ear and the air-filled space of the middle ear.

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PLF appear in the disrupted tissues of the round or oval window and in fractured bony labyrinth or minor fissures that occur after head trauma or barotrauma or after chronic inflammation. They have also been reported to develop spontaneously [Jackler and Brackmann, 2005]. The primary manifestations of perilymph fistulization are sudden or progressive fluctuating sensorineural hearing loss and vertigo. Other symptoms that may be present include tinnitus, disequilibrium and aural fullness. However, the symptoms are not characteristic, especially in cases that have no history of trauma, and so PLF might be confused with idiopathic sudden sensorineural hearing loss, Menière's disease or vestibular neuronitis [Fitzgerald, 2001; Maitland, 2001].

PLF was first proposed as a clinical entity more than a century ago, yet it remains a topic of controversy, especially regarding the occurrence of spontaneous PLF. It is also known that although several potential pathways exist between the perilymphatic space and the middle ear, actual leaking of fluid can be difficult or impossible to prove. The conventional gold standard of PLF detection is the intraoperative visualization of perilymph leakage, which ostensibly confirms the existence of PLF. If the patient does not have PLF, leakage will not be detected. However, the surgical procedure itself invites seepage and bleeding, which accumulates in the concave-shaped round and oval window niches, and this could be misinterpreted as perilymph leakage [Nomura, 1994]. The difficulty of making a definitive diagnosis of PLF has caused a long-standing debate regarding its prevalence, natural history, management and even its very existence [Hughes et al., 1990; Schuknecht, 1992; Friedland, 1999].

This has led to a series of research efforts to identify an endogenous marker of perilymph [Bassiouny et al., 1992; Thalmann et al., 1994; Olaf et al., 2005a, 2005b] or exogenous substances such as intrathecal fluorescein [Gehrking et al., 2002], which might be used to diagnose PLF. Although beta2-transferrin was thought to be a marker, a more recent study showed that, because of the relative amount of serum and perilymph in a mixed sample, electrophoretic separation of the transferrin variant might not be diagnostic [Rauch, 2000]. To date, there is no clinically relevant biochemical marker for perilymph leakage.

Previously, by proteomic analysis of inner ear proteins we found unique properties of cochlin, encoded by the *COCH* gene, mutated in DFNA9, in hereditary hearing loss [Robertson et al., 1998, 2006]. We detected 3 cochlin isoforms (p63s, p44s and p40s) in the inner ear tissue and a short 16-kDa isoform, cochlin-tomoprotein (CTP), in the perilymph [Ikezono et al., 2001, 2004]. An analysis of the isoform structure suggested that the short 16-kDa isoform can be produced by proteolytic cleavage of full length cochlin, and our recent study on splicing variants of cochlin mRNA confirmed this (submitted elsewhere). Therefore we named it 'cochlin-tomoprotein' (*tomo* meaning 'cut' in Greek). Since cochlin was found to be highly specific to the inner ear, we speculated that CTP might also be specific to the perilymph.

CTP was detected in all 20 perilymph samples. By contrast, CTP was not detected in any of the 77 body fluid samples of serum, cerebrospinal fluid (CSF) and saliva. Here, we describe the specificity of CTP expression in perilymph, and discuss the future clinical application of CTP as a diagnostic marker of PLF. CTP has the potential to be a biochemical marker to allow a definitive diagnosis of the etiology of PLF-related hearing loss and vestibular disorders.

Methods

Collection and Processing of Body Fluid Samples

For the assessment of the specificity of CTP expression in body fluids, we collected perilymph during translabyrinthine vestibular schwannoma surgery, stapedectomy for otosclerosis or cochleostomy for cochlear implant surgery. We collected serum and saliva from normal controls. CSF was purchased from Biotech (Valley Center, Calif., USA). The CSF had been collected from consenting donors at an FDA licensed and registered facility. No adverse events were observed during sample collection. The samples were centrifuged at 1250 *g* for 1 min and the supernatants were frozen and stored at –80°C until use. All patients gave their full informed consent and the study was approved by the ethics committee of Nippon Medical School.

Analysis of CTP Expression by Western Blot Analysis

Two micrograms of perilymph, serum, saliva or CSF were mixed with 5 μl of sample buffer (0.188 M Tris buffer, 2.29 mM SDS, 30% glycerol and 15% 2-mercaptoethanol) after normalization per average protein concentration (perilymph 200 mg/dl, plasma 7000 mg/dl, CSF 40 mg/dl and saliva 100 mg/dl) [Thalmann et al., 1994; Mata et al., 2004], then analyzed by Western blot.

For Western blot analysis, the rabbit polyclonal anti-CTP antibody (formerly anti-LCCL-CA*b*) was prepared as previously described (fig.1) [Ikezono et al., 2004]. In brief, 14-mer peptide (LSRWSASFTVTKGK) corresponding to residues 114–127 in the LCCL domain was used to generate antibody. We added cysteine residues to the C termini of the peptides to permit coupling of the peptides to KLH as a carrier protein for immunization. Rabbits were immunized by repeated subcutaneous injections of the KLH-coupled peptides. The serum was purified by a protein A column, followed by peptide-affinity chromatography. The specificity of the antibodies for the corresponding antigenic peptides

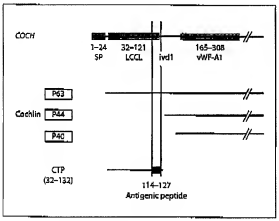

Fig. 1. Representation of the *COCH* gene, cochlin and CTP. The top line denotes the deduced amino acid sequence of human *COCH*, showing the positions of the signal peptide (SP), the Limulus factor C, cochlin and the late gestation lung protein Lgl1 domain (LCCL), the intervening domains 1 (ivd1) and the von Willebrand factor type A-like domains 1 (vWF-A1). The middle lines depict the cochlin isoforms, p63a, p44a and p40a, expressed in inner ear tissue. The bottom line depicts the cochlin-tomoprotein (CTP) isoform expressed in the perilymph. The black bar indicates the location of the antigenic peptide and vertical lines represent the alignment of antigenic peptide and cochlin isoforms. Numbers are the corresponding amino acid sequence of human cochlin. The exact N and C terminal sequence of CTP is not yet known. However, a putative CTP sequence predicted from our previous study [Ikezono et al., 2004] for amino acid residues 32–132 was used to measure the detection limit of hrCTP.

Table 1. Specificity of CTP expression in body fluids

Sample	Total	CTP positive	CTP negative
Perilymph			
Vestibular Schwannoma surgery	9	9	0
Stapedectomy	9	9	0
Cochlear implant	2	2	0
Total	20	20	0
Body fluids			
Serum	28	0	28
CSF	20	0	20
Saliva	29	0	29
Total	77	0	77

was confirmed by dot blot analysis and a peptide absorption test (data not shown).

Samples were loaded onto 15% polyacrylamide gels and transferred onto PVDF membranes. Membranes were blocked overnight at 4°C in 5% skim milk and 0.2% polyoxyethylenesorbitan (Tween-20) dissolved in PBS (pH 7.5). Membranes were then incubated in PBS containing 1% skim milk and 0.1% Tween-20 for 2 h at room temperature with the primary antibody (anti-CTP antibody) diluted at 1:1000. After washing with 0.1% Tween-20 in PBS, membranes were incubated for 1 h at room temperature with horseradish peroxidase-labeled goat anti-rabbit IgG antibody (Dako, Tokyo, Japan) diluted at 1:1000 in the same buffer used for the primary antibody reaction. They were washed again and the reaction was developed with a chemiluminescence reaction kit (ECL Advance; GE Healthcare, Amersham, UK) and then analyzed by an LAS-3000 image analyzer (Fuji Film, Tokyo, Japan).

Detection Limit of the Recombinant Human (rh)CTP by Western Blot

The recombinant human (rh)CTP was produced to measure the detection limit of the Western blot. The exact N and C terminal sequence of CTP is not yet known. However, a putative CTP sequence predicted from our previous study [Ikezono et al. 2004] for the positions 101–403 of the cDNA, corresponding to amino acid residues 32–132 (fig. 1), was amplified by PCR from a human expressed sequence tag clone, Image ID 27789 (Kurabo, Japan). According to the manufacturer's protocol, rhCTP was produced at a final concentration of 0.17 mg/ml using pCR/T7/TOPO/TA expression kits (Invitrogen, Tokyo, Japan). Serially diluted rhCTP were tested for assessment of the detection limit. Serially diluted perilymph samples were also tested to establish the detection limit of CTP.

Results

CTP Expression in Body Fluid Samples

Perilymph from 9 vestibular schwannoma surgery, 9 stapedectomy and 2 cochlear implant patients were all positive for CTP (table 1, fig. 2). However, CTP was not detected in any of the 77 body fluid samples (28 serum, 20 CSF and 29 saliva). These results show that CTP is a perilymph specific protein.

Detection Limit of the Recombinant Human (rh)CTP by Western Blot

The molecular weight of rhCTP exactly matched that of native CTP (16 kDa) on Western blot (fig. 2c). This rhCTP is suitable for future use as a spiked standard when we test the clinical samples by Western blot. The detection limit of the rhCTP was between 0.27 and 0.14 ng/well (fig. 3). Two serially diluted perilymph samples were tested to show the detection limit. The average minimum detection limit of CTP from perilymph was 0.022 μl peri-

lymph/well (data not shown). This detection limit could be good for the clinical use of CTP as a diagnostic marker of PLF.

Discussion

PLF has some proven etiologies, and these must be considered in the appropriate settings (e.g. osseous labyrinth fracture, blast explosion, middle ear trauma, ear surgery such as post-stapedectomy) [Shea, 1963; Schuknecht, 1969; Strohm, 1986; Jackler et al., 1987; Fitzgerald, 1996; DePalma et al., 2005; Jackler and Brackmann, 2005]. A PLF should be considered in pediatric patients with recurrent meningitis, and middle ear exploration should be pursued [Jackler et al., 1987; Reilly, 1989]. PLF without perilymph leakage has also been established as a clinical entity, as seen in superior canal dehiscence syndrome or semicircular canal fistula caused by cholesteatoma [Minor, 2003], which can be diagnosed by high resolution CT scan. Other etiologies have also been proposed to cause PLF (which require detection of perilymph leakage detection to establish a diagnosis), such as a traumatic or barotraumatic event resulting in disruption of the membranes of the round and/or oval window(s) or leakage from minor fissures [Kohut et al., 1986; Goodhill, 1971; Klingmann et al., 2007].

There is no established diagnostic test with enough sensitivity and specificity to identify the presence or absence of perilymph leakage. This has made it difficult to establish criteria to determine when surgical exploration might be indicated. Additionally, there are no universally accepted criteria to confirm diagnosis at surgery; the determination of perilymph leakage is still a subjective decision of the surgeon. The presence of clear fluid in the middle ear at the time of surgery may represent perilymph or may be CSF, serum, seepage, or local anesthetic. No reliable and accurate test is currently available to distinguish these fluids from one another [Nomura, 1994].

The *COCH* gene mutated in DFNA9, an autosomal dominant hereditary sensorineural hearing loss and vestibular disorder, encodes cochlin. Eleven missense mutations and 1 in-frame deletion have been reported (http://web01.ua.ac.be/hhh). By immunohistochemistry on the DFNA9 temporal bone sections, we have shown cochlin staining of the characteristic cochlear and vestibular acellular eosinophilic deposits, indicating aggregation of cochlin in the same structures in which it is normally expressed [Robertson et al., 2006].

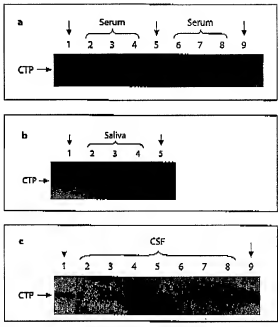

Fig. 2. Western blot analysis for CTP expression in body fluids. The expression of CTP in various body fluids was analyzed by Western blot using the anti-CTP antibody. Two micrograms of perilymph, serum, saliva or CSF was mixed with 5 μl of sample buffer after normalization per protein concentration (perilymph 200 mg/dl, plasma 7000 mg/dl, CSF 40 mg/dl, saliva 100 mg/dl). CTP expression (16 kDa) was detected in the perilymph, but not in the serum, CSF or saliva. CTP was detected only in perilymph samples. **a** Lanes 1, 5 and 9 (arrows) contain perilymph, all others contain serum. **b** Lanes 1 and 5 (arrows) contain perilymph, all others contain saliva. **c** Lane 1 (arrowhead) contains rhCTP (0.27 ng), lane 9 (arrow) contains perilymph, and all others contain CSF. The molecular weight of rhCTP exactly matched that of native CTP (16 kDa) on Western blot.

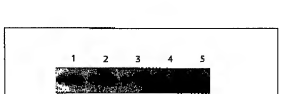

Fig. 3. Detection limit of the rhCTP by Western blot. rhCTP was produced using pCR/T7/TOPO/TA expression kits. Serially diluted rhCTP were tested for assessing the detection limit. The detection limit of the rhCTP was between 0.27 and 0.14 ng/well. Amount of protein per lane: lane 1 = 1.08 ng; lane 2 = 0.54 ng; lane 3 = 0.27 ng; lane 4 = 0.14 ng; lane 5 = 0.07 ng.

The function of cochlin has not yet been fully elucidated, but there is a line of evidence that cochlin is a very important protein for inner ear function. Cochlin is an extracellular matrix protein, and a major constituent of the inner ear, comprising 70% of the non-collagenous inner ear protein [Ikezono et al., 2001]. A spatiotemporal expression study of cochlin suggests that it may be deeply related to the maturation of inner ear function [Shindo et al., 2008]. Cochlin has unique isoforms. We reported 3 cochlin isoforms (p63s, p44s and p40s) expressed in the inner ear tissue composed of 16 different protein spots, with charge and size heterogeneity. A 16-kDa short isoform of cochlin, CTP, was identified not in the membranous labyrinth but *only* in the perilymph [Ikezono et al., 2001, 2004]. Full-length cochlin, p63s, has 2 functional domains, an LCCL module of unknown function and vWF-A like domain which might work as a binding domain to collagen. In fact, electron microscopic study revealed the co-localization of cochlin and type II collagen [Mizuta et al., 2008]. Interestingly, the whole molecule of CTP is composed of only an LCCL domain and most of the mutations of cochlin reported in DFNA9 are located in this domain. CTP could be an important molecule by which to understand the function of cochlin and the pathophysiology of DFNA9.

The mechanisms for the formation of these isoforms are unknown, but it is speculated that CTP is cleaved from full-length cochlin p63s by inner ear cells and then secreted into the endo- or perilymphatic space in the inner ear. Alternatively, CTP may be directly coded from a unique *COCH* gene splice variant or from a *COCH* homologue [Kommareddi et al., 2007]. The *COCH* gene was initially isolated by subtractive hybridization and Northern blot. A microarray expression profile analysis showed that *COCH* is preferentially expressed in the inner ear [Robertson et al., 1994; Abe et al., 2003]. Our previous study using rat organs showed that *COCH* gene is expressed preferentially in the inner ear and that expression of full-length cochlin p63s is specific to the inner ear [Li et al., 2005]. Therefore, it is rational to speculate that CTP expression could also be inner ear specific, and indeed CTP is a perilymph specific protein as shown in the present study. The detection limit of the rhCTP was between 0.27 and 0.14 ng/well, and the average detection limit of perilymph was 0.022 µl/well on Western blot analysis. This detection limit could be good for the clinical use of CTP as a diagnostic marker of PLF. We are presently conducting CTP detection tests on PLF suspected cases and evaluating the diagnostic accuracy of the test. The ultimate purpose of this test is to be able to detect the presence of leaked perilymph in the middle ear cavity preoperatively in the outpatient clinic or intra-operatively during exploratory tympanotomy. The leaked perilymph in the middle ear can be microliters in quantity. We collect this minute volume of perilymph by lavaging the middle ear cavity 4 times with 0.3 to approximately 0.4 ml of saline and recovering the fluid. Native CTP in the lavage could be detected by Western blot.

A number of authors have suggested the identification of an endogenous perilymph marker substance. Thalmann et al. [1994] gave a detailed analysis and comparison of plasma, perilymph and CSF protein, using high-resolution 2-dimensional polyacrylamide gel electrophoresis, combined with amino acid sequencing. The majority of proteins were found to be present in perilymph at levels in basic agreement with the total protein gradient between perilymph and plasma (1:35). However, high-density lipoprotein-associated apolipoprotein apo D detected in perilymph was at a 2.1-fold higher concentration than in plasma. This characteristic might make it a marker for PLF.

Previously tested candidate markers such as beta-2 transferrin, Beta-trace protein (prostaglandin D synthase), or intrathecal fluorescein, are markers of CSF leakage. An electrophoretic assay of middle ear fluid for the presence of beta-2 transferrin, a protein unique to CSF, aqueous humor and human perilymph was introduced for the diagnosis of PLF [Bassiouny et al., 1992; Buchman et al., 1999]. In children suspected to have PLF, beta-2 transferrin was detected in 6 of 9 operated ears, all 10 control patients were negative [Weber et al., 1994]. Although this technique holds promise, the dilutional effect of sample handling in preparation may lower beta-2 transferrin concentration below the detection limits of the assay. Because of the relative amount of serum and perilymph in a mixed sample, electrophoretic separation of transferrin variants may not be diagnostic [Levenson et al., 1996; Rauch, 2000].

Beta-trace protein (prostaglandin D synthase) has been used as a CSF leakage marker [Bachmann et al., 2002]. Since beta-trace protein is detectable in inner ear fluids in an even higher concentration than in CSF, it is a potential marker for perilymph leakage. However, there are some drawbacks that are pointed out by the author: the normal concentration of beta-trace protein in perilymph and proper cut-off of perilymph detection is not known yet, and it is impossible to distinguish CSF leakage from PLF leakage [Olaf et al., 2005a, 2005b; Risch et al., 2005].

There have been reports of fluorescein use as an exogenous marker for PLF diagnosis. Although it is appealing as a marker, visual detection of faint fluorescence in a small-volume middle ear fluid sample may be subjective, which is the same drawback in conventional visual detection of perilymph leakage. The wide, rapid distribution throughout all physiological fluid compartments makes it an unreliable marker substance in the differential diagnosis of PLF [Poe et al., 1993; Rauch, 2000; Gehrking et al., 2002].

CTP is the first substance that is present in the perilymph, but not in other body fluids such as the CSF, serum and saliva. Therefore, it can be a sensitive biochemical marker for perilymph leakage. Once a well-standardized CTP detection test is established, it could be a definitive objective test for the diagnosis of PLF.

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GENOMICS 23, 42–50 (1994)

Isolation of Novel and Known Genes from a Human Fetal Cochlear cDNA Library Using Subtractive Hybridization and Differential Screening

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We used a combination of subtractive hybridization and differential screening strategies to identify genes that may function normally in hearing and, when mutated, result in deafness. A human fetal cochlear (membranous labyrinth) cDNA library was subtracted against total human fetal brain RNAs by an avidin–biotin-based procedure to enrich for cochlear transcripts. Subtracted cochlear clones were differentially screened with ^{32}P-labeled total cochlear and total brain cDNA probes. Sequence analysis of clones that hybridized more intensely with cochlear than with brain cDNA probes revealed some previously characterized genes, including mitochondrial sequences, collagen *type I* α-*2* (*COL1A2*), collagen *type II* α-1 (COL2A1), collagen type III α-1 (COL3A1), spermidine/spermine N^1-acetyltransferase (SAT), osteonectin (SPARC), and peripheral myelin protein 22 (PMP22). Also identified were clones that are potential novel cochlear genes. Northern blots of cochlear and brain RNAs probed with COL1A2, COL2A1, COL3A1, SAT, SPARC, PMP22, and a novel sequence, designated Coch-5B2, confirm results of the subtractive procedure by showing preferential cochlear expression. A number of these genes serve structural or regulatory functions in extracellular matrix or neural conduction; defects in some of these genes are associated with disorders involving hearing loss. Partial sequence analysis of Coch-5B2 reveals a von Willebrand factor type A-like domain in this cDNA. To assess the cochlear specificity of Coch-5B2, a Northern blot panel of 14 human fetal tissue RNAs was probed with Coch-5B2, showing differential expression of this novel gene in the cochlea. © 1994 Academic Press, Inc.

Sequence data from this article have been deposited with the GenBank/EMBL Data Libraries under Accession No. U09203.

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INTRODUCTION

Despite advances in the understanding of clinical aspects of hearing and deafness (Nance and Sweeney, 1975; Bieber and Nance, 1979; Bieber, 1981) and studies of cellular and biophysical mechanisms of inner ear hair cell function (Hudspeth, 1989; Teas, 1989), very little is known about the genes and molecular events involved in hearing. Genetic heterogeneity in syndromic and nonsyndromic deafness (Konigsmark and Gorlin, 1976; McKusick, 1992) suggests interaction of many genes in the complex process of hearing (Bodurtha and Nance, 1987).

To study the molecular genetics of hearing and deafness, we have constructed a human fetal cochlear (membranous labyrinth) cDNA library and performed *subtraction* and *differential screening* for enrichment and isolation of cochlear messages. Many approaches involving these molecular methods have used parallel systems in which induced vs noninduced (Almendral *et al.*, 1988; Friedman and Weissman, 1991; Mohn *et al.*, 1991; Owens *et al.*, 1991; Benvenisty *et al.*, 1992), disease vs nondisease (Duguid *et al.*, 1988; Basset *et al.*, 1990; Schweinfest *et al.*, 1990), aging vs nonaging (Murano *et al.*, 1991), or various stages of differentiation and development (Hara *et al.*, 1991; Rothstein *et al.*, 1992) have been compared to identify tissue-specific genes. Tissue-specific genes have also been identified using nonparallel systems such as subtraction of *human retina and lymphoblastoid cell line libraries* (Swaroop *et al.*, 1991; Geiser and Swaroop, 1992). We have chosen a nonparallel and broad approach by subtraction and differential screening of human cochlea with brain, which expresses a large number of tran-

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scripts (Sutcliffe, 1988; Adams *et al.*, 1992), to reduce the number of common housekeeping genes. We have found this strategy to be valuable in enriching for cochlear messages.

A large variety of different techniques have been used successfully to enrich for tissue-specific transcripts. We describe the methods that we have chosen and modified for our system and available resources to generate a subtracted cochlear cDNA library and subsequently to screen for preferentially expressed clones. Our subtracted library is the only human cochlear cDNA library constructed to date to our knowledge from this rare tissue source, providing a renewable resource for continued isolation of genes that may serve critical functions in hearing.

We report isolation of one novel sequence as well as several known genes expressed preferentially in cochlea as compared to brain. In addition, we discuss expression of the previously known genes, a number of which encode structural or regulatory components of extracellular matrix or function in neural conduction. Some of these genes have also been previously associated with disorders showing sensorineural hearing loss, revealing new information on their possible roles in the hearing process.

MATERIALS AND METHODS

Construction of Directional cDNA Library and PCR Analysis of Insert Sizes

One hundred seventy-three membranous labyrinths (cochlea) were obtained from human fetuses at 16–22 weeks developmental age in accordance with guidelines established by the Human Research Committee at the Brigham and Women's Hospital. Total cellular RNAs were extracted (Chirgwin *et al.*, 1979) and poly(A)$^+$ RNAs were selected (Aviv and Leder, 1972) from approximately 500 μg of total cellular cochlear RNAs. An oligo(dT)-primed, directional cDNA library was constructed in the Uni-ZAP XR vector (Stratagene, La Jolla, CA); cochlear cDNAs were size selected at $\geq$400 bp.

To assess size distribution of inserts in the cochlear cDNA library, XL1-Blue *Escherichia coli* were infected with phage and plated out at a low density to allow for separation of individual plaques. One hundred six plaques were randomly selected and inserts amplified by PCR using T3 and T7 primers (Stratagene). Products of the amplification were electrophoresed in 1% agarose gels to visualize insert sizes.

Subtractive Hybridization

A schematic representation of this procedure is presented in Fig. 1.

Production of single-stranded phagemid by in vivo excision. Single-stranded cDNAs representing the antisense orientation of the cochlear mRNAs were obtained from the directionally cloned fetal cochlear library by *in vivo* excision or "rescue" of Bluescript phagemid from Uni-ZAP XR vector using a modification of the manufacturer's protocol: 1 ml of XL1-Blue *E. coli* resuspended at OD_{600} of approximately 1.5 in SM buffer (20 m*M* Tris–HCl, pH 7.5, 100 m*M* NaCl, 10 m*M* $MgSO_4$, 0.01% gelatin) was coinfected with 1.5 μl of amplified cochlear cDNA library ($2.5 \times 10^7/\mu$l) and 4 μl of R408 helper phage ($1 \times 10^6/\mu$l). Mixtures were adsorbed at 37°C for 15 min, followed by addition of 20 ml L-broth, incubation at 37°C, and agitation at 180 rpm for 5 h. Six coinfections were performed and pooled for large scale isolation of single-stranded phagemid.

Cultures were spun at 12,000*g* for 15 min, and supernatant containing single-stranded DNAs was collected. Single-stranded DNAs were PEG precipitated, phenol–chloroform extracted, and ethanol precipitated. A portion of single-stranded cochlear cDNA was not subtracted with any sequences, as a comparison to the subtracted cochlear cDNA, to assess the degree of enrichment for cochlear message.

RNA preparation and photobiotinylation. As a source of "driver" for subtractive hybridization (Fig. 1), total cellular RNAs were extracted (Chirgwin *et al.*, 1979) from second trimester human fetal brain cortex and poly(A)$^+$ RNAs were selected (Aviv and Leder, 1972). Two rounds of photobiotinylation of poly(A)$^+$ brain RNAs were performed using Photoprobe Biotin (Vector Laboratories, Burlingame, CA) according to previously described methods (Welcher *et al.*, 1986; Sive and St. John, 1988). Samples were then ethanol precipitated and resuspended in HE buffer (10 m*M* Hepes, pH 7.5, 1 m*M* EDTA).

Subtractive hybridization and avidin binding. The excised cochlear cDNA library (tracer) and the photobiotinylated brain RNAs (driver) were hybridized in a 10:1 excess of brain RNAs. Subtractive hybridization was performed according to a modification of previously reported methods (Duguid *et al.*, 1988; Schweinfest *et al.*, 1990). Approximately 2 μg of single-stranded rescued phagemids from the human fetal cochlear cDNA library, 20 μg of photobiotinylated poly(A)$^+$ brain RNAs, and 3 μg each of poly(dA) and poly(dC) (Pharmacia) were ethanol precipitated, resuspended in 10 μl of HE buffer, and added to 10 μl of 2× hybridization buffer (50 m*M* Hepes, pH 7.5, 1.5 *M* NaCl, 10 m*M* EDTA, 0.2% SDS). The hybridization mixture was heated at 100°C for 1 min and incubated for 24 h submerged in a 68°C waterbath. The hybridization reaction was diluted 10-fold by adding 180 μl of binding buffer (100 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl), incubated at 55°C for 5 min, added to 250 mg of rehydrated Vectrex Avidin (Vector Laboratories), and incubated at room temperature for 30 min. Following microcentrifugation and two washes of the Vectrex Avidin matrix with binding buffer, supernatants containing nonbiotinylated cochlea-enriched cDNAs were combined, ethanol precipitated, and resuspended in 20 μl of 5 m*M* Tris–HCl, pH 7.5, 0.1 m*M* EDTA.

Transformation of E. coli. Subtracted cochlear cDNAs were made double-stranded using T3 primer and Klenow (GIBCO/BRL, Gaithersburg, MD). To generate a plasmid cDNA library from subtracted cochlear sequences, 10 μl (of a total of 50 μl) of the double-stranded subtracted cochlear cDNAs were used to transform XL1-Blue competent *E. coli* (Stratagene) according to the manufacturer's protocol. White colonies were picked into 96-well microtiter trays containing 200 μl ampicillin and stored as glycerol stocks at −80°C as described (Schweinfest *et al.*, 1990). A total of approximately 600 white colonies were generated from two rounds of transformation, each with 10 μl of the double-stranded cochlear cDNA reaction. Parallel transformation of *E. coli* with unsubtracted cochlear cDNAs prepared in the same manner as the subtracted counterpart was performed to obtain a relative estimate of the degree of subtraction.

Differential Screening of Subtracted Cochlear cDNA Library

Differential screening of clones from the subtractive hybridization was performed as a further selection of cochlear cDNAs. Because the number of clones generated to date is approximately 600 (with the potential for another 900), differential screening (Sargent, 1987) was chosen as the next screening step rather than random screening of all clones by Northern blots.

PCR generation of inserts and slot blotting. PCR amplification of cDNA inserts from the subtracted cochlear cDNA library was performed to generate DNA for duplicate slot blots. Five microliters (of 50 μl reaction mixture) of PCR products representing cDNA inserts from the subtracted cochlear cDNA library were visualized on 1% agarose gels. The remaining portion of these products were dena-

tured, neutralized, and applied to nitrocellulose filters using the Bio-Dot SF Microfiltration Apparatus (Bio-Rad Laboratories, Richmond, CA) following the manufacturer's protocol. Equal amounts of insert DNAs were applied on duplicate filters.

cDNA probe synthesis. Two sets of differential screens were performed: one with the first 300 subtracted clones and another with the second 300 subtracted clones. In the first differential screen, approximately 11 μg of fetal cochlea and brain total RNAs were oligo(dT) primed and reverse transcribed separately using 200 units of Superscript Moloney murine leukemia virus (MuMLV) reverse transcriptase (GIBCO/BRL) according to the manufacturer's protocol except for the use of 0.01 m*M* cold dCTP and 60 uCi of [α-^{32}P]-dCTP (6000 Ci/mmol) (Dupont/NEN, Boston, MA). Unincorporated nucleotides were removed using Sephadex-G50 columns (Pharmacia). The RNA template was hydrolyzed with NaOH at 70°C for 20 min and neutralized.

In the second differential screen, approximately 1–2 μg of poly(A)$^+$ RNAs was prepared from fetal cochlea and brain using the Dynabeads magnetic oligo(dT) mRNA purification system (Dynal, Great Neck, NY) following the manufacturer's protocol. Poly(A)$^+$ RNAs were oligo(dT)-primed and reverse transcribed using 40 units of avian myeloblastosis virus (AMV) reverse transcriptase (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's protocol except for the use of 0.02 m*M* cold dCTP and 300 uCi of [α-^{32}P]-dCTP (3000 Ci/mmol). Labeled cDNAs were purified in the same manner as described above for the first labeling reaction.

Hybridization. Slot blot nitrocellulose filters were prehybridized at 42°C and then hybridized with 10^6 cpm/ml of either ^{32}P-labeled cochlea or brain cDNA probes at 42°C for 48 h in 10% dextran sulfate, 4× SSC, 7 m*M* Tris–HCl, pH 7.6, 0.8× Denhardt's solution, 200 μg/ml sonicated herring sperm DNA, 40% formamide, and 0.5% SDS. cDNA probes were denatured at 100°C for 3 min, and the denaturation was quenched on wet ice. Filters were washed in 0.1× SSC, 0.1% SDS at 42°C, prior to autoradiography using XAR-5 film (Eastman Kodak Co., Rochester, NY) and intensifying screens at −80°C.

Nucleotide Sequence Analysis

Partial nucleotide sequence of clones was determined by the dideoxy chain termination method (Sanger *et al.*, 1977) using the Pharmacia sequencing kit. Sequence analysis was performed using the University of Wisconsin Genetics Computer Group software (Devereux *et al.*, 1984). Comparison of sequences to nucleotide sequences deposited in GenBank and EMBL, and to peptide databases (PIR 31.0, SWISS-PROT 20.0, GenPept), was performed using the BLAST Network Service of the National Center for Biotechnology Information (Altschul *et al.*, 1990).

Northern Blot Analysis

Ten micrograms of total cellular RNAs from human fetal membranous labyrinths (cochlea) and human fetal brain was extracted (Chirgwin *et al.*, 1979), electrophoresed in denaturing 1% agarose–formaldehyde gels, and transferred to GeneScreen (DuPont, Wilmington, DE) filters (Thomas, 1980). Prior to transfer, ethidium bromide-stained RNAs were visualized to confirm integrity and concentration. Filters were prehybridized for 2–4 h and hybridized overnight at 42°C in the same solution as described above with ^{32}P-labeled probes (Feinberg and Vogelstein, 1984) corresponding to six known genes, one novel sequence (detailed under Results) and a human β-actin control. Filters were washed in 0.1% SDS in 0.1× SSC at 42 or 60°C prior to autoradiography using XAR-5 film with intensifying screens at −80°C.

RESULTS AND DISCUSSION

Membranous Labyrinth (Cochlear) cDNA Library

During human gestation, the otic vesicle develops from an invagination and thickening of the surface ectoderm, the otic placode, at about 4 weeks developmental age (Smith, 1975; Sadler, 1985; Noden and Van De Water, 1986). At the sixth week, the cochlear duct develops from a tubular out-pocketing from the ventral portion of the otic vesicle. By the end of the eighth developmental week, the cochlear duct has completed the entire two-and-a-half turns and the organ of Corti and the stria vascularis develop in the wall of the cochlear duct. Differentiation is well under way by the end of the first trimester (12 weeks) and is essentially complete by the end of the second trimester (24 weeks).

Our histologic sections of cochlea at 17–19 developmental weeks show that the organ of Corti is in the process of maturation with discernible outer hair cells, and basilar and tectorial membranes. Also at this stage, the developing stria vascularis and spiral ligament can be seen as well as spiral ganglion cells, which are clearly distinguishable but smaller than adult cells. By approximately 22 weeks of development the following structures are visible: peripheral dendrites of the cochlear division of the VIIIth cranial nerve, the basilar membrane, the organ of Corti with inner and outer hair cells and their supporting cells, and the tunnel of Corti (Khetarpal *et al.*, 1994).

Histology of the fetal membranous labyrinth along with evidence of fetal response to sound stimuli *in utero* during late second trimester (Birnholz and Benacerraf, 1983; Crade and Lovett, 1988) are indicative of the extensive degree of differentiation and development of the cochlea represented in the cDNA library, and therefore indicate that many genes in this library are likely to be involved in the auditory process. Furthermore, genes playing roles in the temporal development of the inner ear at this stage may be expressed and any genes characterized in this library may be relevant in a developmental context.

The cDNA library was constructed from membranous labyrinths (cochlea) from human fetuses at 16–22 weeks developmental age. Careful microscopic dissections were performed to include only membranous portions of the labyrinth and to exclude surrounding bony and cartilaginous elements. Most cochlea were obtained from fetuses at 17–20 weeks of developmental age, with approximately 54% of cochlea from fetuses at 19 developmental weeks or greater.

In the construction of the cDNA library, 3.8 million primary plaques were obtained with less than 5% nonrecombinants. The estimated titer was 4.8×10^6/ml for the unamplified library and 2.5×10^{10}/ml after amplification. We performed a preliminary characterization of insert sizes of the library by random selection of 106 clones and electrophoresis of insert DNAs in agarose gels. The size distribution in kilobasepairs of the randomly selected cloned inserts is 37% at <0.5 kb, 56% ranging between 0.5 and 1 kb, and 7% greater than 1 kb. The relative paucity of cDNAs greater than 1 kb may reflect postmortem autolysis of cochlear RNAs obtained from human fetuses and the technical difficulty

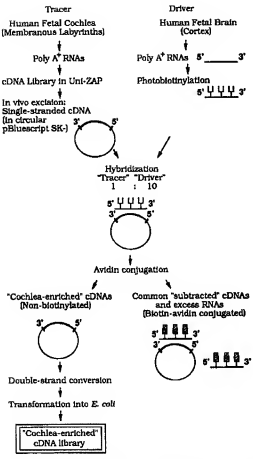

FIG. 1. Schematic representation of the subtractive hybridization procedure for construction of the "cochlea-enriched" cDNA library.

of performing dissections of membranous labyrinths from temporal bones.

Subtractive Hybridization

Our goal is to identify genes from our human fetal cochlear cDNA library that are preferentially or specifically expressed in cochlea, as they may play an important role in the process of hearing. We have pursued subtractive hybridization as a method to enrich for cochlear-specific messages to isolate potential genes of interest from genes commonly expressed in other tissues. The subtraction scheme is presented in Fig. 1. We have chosen brain (cortex) as "driver," the sequences of which are subtracted from membranous labyrinths (cochlea), the "tracer." Brain was selected for the driver because it is a highly complex tissue in both number and diversity of mRNAs transcribed (Sutcliffe, 1988; Adams *et al.*, 1992). We have used mRNA isolated directly from brain because of the availability of brain tissue to us and the potential for better representation of messages compared to that from synthetic RNA generated from a brain cDNA library.

The *in vivo* excision step in our protocol yielded small amounts of single-stranded Bluescript containing cochlear cDNAs and much larger amounts of helper phage DNA. This relationship held true with numerous changes in variables in the protocol, including multiplicity of infection of phage to bacterial cells, ratio of helper phage to library, incubation time, harvesting of cells, and isolation of single-stranded DNA. The presence of helper phage DNA along with the rescued library did not appear to interfere with any subsequent steps in the subtraction protocol. In addition, the relatively small amount of rescued single-stranded library was sufficient to generate an estimated 1500 white colonies after subtraction, double-stranding, and transformation from a starting amount of approximately 2 μg of rescued DNA.

To estimate the degree of enrichment for cochlear messages after subtraction, a portion of the *in vivo* excised cochlear cDNAs was set aside *not* to be subtracted. These unsubtracted cDNAs were double-stranded and used to transform *E. coli* to generate a plasmid library in the same manner as was carried out with the subtracted cDNAs. The number of colonies obtained from unsubtracted cDNAs was approximately 20 times higher than that obtained from the subtracted library, suggesting a 20-fold enrichment for cochlear messages from subtraction.

Differential Screening and Identification of Preferentially Expressed Cochlear cDNAs

To identify cDNAs in our subtracted library that are preferentially or specifically expressed in human fetal cochleas, we chose to screen duplicate slot blots of subtracted cDNAs differentially with radiolabeled cDNA probes generated from cochlea and brain. We chose this extra step of screening rather than testing the specificity of every subtracted clone directly by Northern analysis because of the number of clones (estimated 1500) that came through the subtraction. We analyzed approximately 600 clones by differential screening. Differences in intensity varied, and there were no absolute positive or negative signals on slot blots, as also previously noted (Owens *et al.*, 1991); this may reflect a high background of nonspecific hybridization to the excess amount of insert DNA by cDNA probes of very high complexity. In addition, the brain expresses a large and complex variety of mRNAs such that it is possible that some expression of a gene relevant to cochlea may be detectable in the brain. Therefore, clones that showed any intensity differences in their hybridization with cochlea vs brain probes were considered potential differentially expressed sequences.

Because of paucity of cochlear RNA, we chose sequencing rather than Northern analysis as the initial screening of cochlear clones. Twenty-six clones that hybridized more intensely with cochlear cDNA than with brain cDNA probes on the differential slot blots were

sequenced and compared to all sequences in the GenBank and EMBL databases. Sixteen clones were identified as previously characterized human genes, reflected by a nucleotide homology of ≥95%.

Four clones matched mitochondrial sequences (including mitochondrial tRNAs and cytochrome c oxidase subunit II). Because our library was synthesized from oligo(dT)-primed poly(A)$^+$ RNAs, and mammalian mitochondrial RNAs can be polyadenylated (Attardi and Schatz, 1988), the presence of mitochondrial transcripts in the library is not unexpected. Also, the finding of these messages in greater amounts in cochlea may be a reflection of the abundance of this organelle in this tissue with highly metabolically active cells such as hair cells of the organ of Corti and those of the stria vascularis. Additionally, in light of recent discoveries of mitochondrially inherited diabetes mellitus with deafness due to deletion (Ballinger *et al.*, 1992) or point mutation (van den Ouweland *et al.*, 1992) in the mitochondrial genome, the relationship of the mitochondrion to the tissue-specific manifestation of disease is an interesting finding in the context of hearing.

Nine clones from the differential screen represent members of the human collagen family of genes: seven are collagen type I α-2 (COL1A2), one is collagen type II α-1 (COL2A1), and one is collagen type III α-1 (COL3A1). Other known sequences isolated from the differential screen are human spermidine/spermine N^1-acetyltransferase (SAT), human SPARC (also known as osteonectin), and human peripheral myelin protein 22 (PMP22). The remaining 10 clones selected from the differential screen did not match any known genes in the GenBank or EMBL databases and may represent novel genes.

Northern Analysis

Northern blot analysis was the next step in our characterization of clones to confirm results of subtractive hybridization and differential screening, to show degree of specificity of these sequences to cochlea, and to ascertain size and number of transcripts. Cochlea and brain RNAs were probed to compare the level of expression in the two tissues. It is interesting to note that clones showing very small differences in intensity on slot blot hybridization with cochlea vs brain probes (Fig. 2B) showed much higher intensity differences in expression on Northern blots (Fig. 2A). This is most probably a reflection of higher sensitivity of Northern blots as compared to our differential screen and indicates the potential to select additional differentially expressed sequences by increasing specificity and decreasing background in this screen.

By Northern analysis, seven clones have been found to be expressed at higher levels in cochlea as compared to brain (Fig. 2A), confirming results of the differential screen: COL1A2, COL2A1, COL3A1, SPARC, spermidine/spermine N1-acetyltransferase (SAT), peripheral myelin protein 22 (PMP22), and an unknown clone, designated Coch-5B2, which does not match any known gene in the GenBank and EMBL data bases. This clone is of particular interest as it represents a novel cochlear gene. Two other novel sequences chosen from the differential screen were expressed in approximately equal amounts in cochlea and brain by Northern blot analysis (data not shown). Because of the lower sensitivity of the differential screen, we expect false positives, as also previously reported (Owens *et al.*, 1991). On five Northern blots of other novel sequences, we have seen either no detectable message or nonspecific hybridization to 28S and 18S ribosomal RNAs; further characterization of these clones may be undertaken by using poly(A)$^+$ RNA on Northerns or by RNase protection assays.

High levels of expression of collagen genes COL1A2, COL2A1, and COL3A1 in the cochlea as compared to the brain are shown in Fig. 2A; message sizes correspond to those previously reported (Myers *et al.*, 1981, 1987; Sandberg and Vuorio, 1987). Collagens are known to serve important structural roles in extracellular matrix (ECM) of a variety of tissues. Mutations in collagen genes result in multisystemic disorders that may include hearing loss: COL1A2 in osteogenesis imperfecta (Byers, 1989; Kuivaniemi *et al.*, 1991) and COL2A1 in some patients with Stickler syndrome (Ahmad *et al.*, 1991; Winterpacht *et al.*, 1993), Kniest dysplasia (Winterpacht *et al.*, 1993), and spondyloepithelial dysplasia congenita (Tiller *et al.*, 1990). *In situ* hybridization of COL1A2 (Khetarpal and Morton, 1993) and *in situ* hybridization and immunohistochemistry of COL2A1 in cochlea (Khetarpal *et al.*, 1994) have revealed expression of both of these genes in cells other than those of connective tissue elements.

SPARC, also known as osteonectin, encodes a Ca^{2+} binding secreted protein which is acidic and rich in cysteine. Transcript sizes of approximately 2.2 kb (major band) and 3.0 kb (minor band) seen on Northern blot (Fig. 2A) are in agreement with reported human SPARC mRNA sizes (Swaroop *et al.*, 1988). Furthermore, very low level expression of SPARC message in brain tissue has also been previously reported (Mason *et al.*, 1986a; Nomura *et al.*, 1988), confirming results of our subtraction and differential screen. Unlike many ECM-associated proteins, SPARC has been shown to possess "anti-attachment" properties in a variety of cells (Sage and Bornstein, 1991), and unlike collagens, SPARC is not a structural component of the ECM but rather may play a regulatory role in its formation (Mason *et al.*, 1986b). High levels of SPARC transcript in the fetal membranous cochlea may indicate an important role of this gene in cochlear development, maturation, or function in terms of a variety of roles in ECM formation or remodeling.

Spermidine/spermine N^1-acetyltransferase (SAT) (Casero *et al.*, 1991; Xiao *et al.*, 1991) is the rate-limiting enzyme in the catabolism of polyamines that may

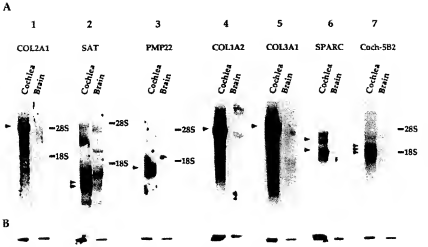

FIG. 2. (A) Autoradiograph of Northern blots of 10 μg of total RNAs extracted from human fetal cochlea (membranous labyrinths) and human fetal brain hybridized with probes selected from the differential screen: (1) COL2A1 (collagen type II α-1), (2) SAT (spermidine/spermine N^1-acetyltransferase), (3) PMP22 (peripheral myelin protein 22), (4) COL1A2 (collagen type I α-2), (5) COL3A1 (collagen type III α-1), (6) SPARC (secreted protein which is acidic and rich in cysteine) (also known as osteonectin), (7) Coch-5B2 (novel gene). A much higher level of expression of these genes is seen in cochlea than in brain. Equal amounts and integrity of RNAs were confirmed by ethidium bromide staining and the presence of 28S and 18S rRNA background bands. Transcripts are indicated by arrowheads to the left of each autoradiograph; the positions of 28S and 18S rRNAs are marked by lines to the right. Message sizes of known genes are in agreement with those previously reported. Transcript sizes of the novel Coch-5B2 sequence (7) are estimated to be 2.9, 2.3, and 2.0 kb and may represent differential use of promoter cap sites or polyadenylation signals, products of alternative splicing or multiple genes. Nonspecific hybridization representing 28S and 18S rRNA background bands is seen in some samples. (B) Autoradiograph of the differential screen showing slot blots containing equal amounts of cDNA inserts from the subtracted cochlea library hybridized with ^{32}P-labeled total cochlear cDNA probes (left slot) or with ^{32}P-labeled total brain cDNA probes (right slot). These clones, which showed slightly higher intensity of hybridization with total cochlear than with total brain cDNA probes on slot blots, were chosen as probes for the corresponding Northern blots shown above each respective set of slot blots. Note much larger intensity differences between cochlea and brain message levels seen on Northerns as opposed to intensity differences seen on slot blots from the differential screening.

play regulatory roles in nucleic acid and protein synthesis and cell division (Metzler, 1977). On Northern blot (Fig. 2A), the specific hybridization seen in cochlea at approximately 1.0 and 1.1 kb is consistent with the reported transcript size, which includes a band spanning 1.1 to 1.3 kb (Xiao *et al.*, 1991).

PMP22 message has been found in Schwann cells of the peripheral nervous system (Snipes *et al.*, 1992). Reported human transcript sizes are 1.8, 1.3, and 0.8 kb (Patel *et al.*, 1992); the 1.8-kb transcript is seen in cochlea (Fig. 2A). Very low level or undetectable message in the brain have been reported (Patel *et al.*, 1992; Snipes *et al.*, 1992), corroborating our Northern blot and subtraction results. Duplication or point mutation in human PMP22 has recently been associated with Charcot–Marie–Tooth disease type 1A (CMT1A) (Matsunami *et al.*, 1992; Patel *et al.*, 1992; Timmerman *et al.*, 1992; Valentijn *et al.*, 1992a, b), an inherited demyelinating sensorimotor neuropathy (Hoogendijk and De Visser, 1991) with hearing loss reported in some patients. High-level expression of PMP22 in the cochlea is interesting in terms of its role in myelination and nerve conduction in this tissue.

We have designated the remaining clone that we have shown to be preferentially expressed in the cochlea as Coch-5B2. Sequence comparison of Coch-5B2 to those in GenBank and EMBL databases shows that Coch-5B2 is not a previously identified gene. Partial sequence analysis of Coch-5B2 identified a von Willebrand factor type A-like domain, suggesting that it may be a novel member of this superfamily of genes. Known proteins in this superfamily have diverse functions, including extracellular matrix assembly, hemostasis, cellular adhesion, and defense mechanisms (Colombatti and Paolo, 1991); one role of this domain may be in the binding of collagen. Northern blot analysis of Coch-5B2 shows three transcript sizes of approximately 2.9, 2.3, and 2.0 kb with the 2.3-kb transcript representing the predominant message (Fig. 2A). These transcripts may represent differential use of promoter cap sites or polyadenylation signals, products of alternative splicing or multiple genes. To assess cochlear-specific expression of Coch-5B2, Northern blot analysis was performed with an extensive panel of human fetal tissues, including cochlea, brain, liver, spleen, skeletal muscle, kidney, lung, skin, thymus, adrenal, small intestine, eye, cartilage, and cultured fibroblasts (Fig. 3). Three transcripts are seen at very high levels in the cochlea, whereas messages are undetectable in other tissues, except for low levels in brain and eye. Hybridization with a human β-actin probe (data not shown), in addition to the presence of 28S and 18S rRNA background

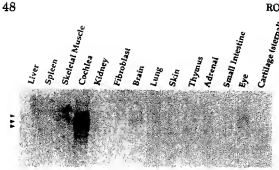

FIG. 3. Autoradiograph of Northern blot of 10 μg of total RNAs extracted from human fetal cochlea, brain, liver, spleen, skeletal muscle, kidney, lung, skin, thymus, adrenal, small intestine, eye, sternal cartilage, and cultured fibroblasts hybridized with the Coch-5B2 probe, a novel cochlear cDNA isolated by subtractive hybridization and differential screening. Differential expression of Coch-5B2 in cochlea as compared to other tissues tested is seen, represented by high levels of three transcripts in the cochlea; very low levels are detected in brain and eye. Ethidium bromide staining of RNAs, hybridization with a human β-actin probe (data not shown), and presence of 28S and 18S rRNA background bands in less stringent washes confirmed integrity, equal loading, and even transfer of RNAs. Transcripts are indicated by arrowheads; the positions of 28S and 18S rRNAs are marked by lines.

bands in less stringent washes, confirmed integrity, equal loading, and even transfer of RNAs. Differential expression of the novel cDNA Coch-5B2 in the cochlea, as compared to a wide variety of human tissues ranging from structural to hematopoietic, to other specialized tissues may indicate an important function for this gene in the cochlea. Furthermore, the finding of expression of Coch-5B2 in cochlea and eye is particularly relevant to disorders involving both of these sensory systems, making Coch-5B2 a candidate gene for the genetically heterogeneous Usher syndrome (Fishman *et al.*, 1983), which includes sensorineural deafness and retinitis pigmentosum.

The subtracted human cochlear cDNA library will provide a valuable reagent to access additional genes that are preferentially expressed in the cochlea. Identification and characterization of both novel as well as previously known genes are of interest in terms of their role in cochlear development, maturation, and function in the hearing process.

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Mapping and Characterization of a Novel Cochlear Gene in Human and in Mouse: A Positional Candidate Gene for a Deafness Disorder, DFNA9

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Previously we identified a partial human cDNA for a novel cochlear transcript, hCoch-5B2 (HGMW-approved symbol D14S564E), using subtractive hybridization techniques. Herein we report isolation and characterization of both human and mouse (D12H14S564E) cDNAs for Coch-5B2. Full-length Coch-5B2 deduced amino acid sequences reveal a very high degree of conservation in the coding region (89% nucleotide and 94% amino acid identity) and a potential signal peptide and two regions of extensive homology to the collagen-binding type A domains of von Willebrand factor, also present in other secreted proteins, including extracellular matrix components. High levels of hCoch-5B2 expression are seen only in human fetal inner ear structures, cochlea, and vestibule, among a large panel of human fetal and adult tissues. Coch-5B2 expression in the mouse is more widespread than in the human, with message detected in mouse adult spleen, cerebrum, cerebellum/medulla, and thymus. In both species very low level expression is detected in total eye. More specifically, mouse retina shows a higher level of mCoch-5B2 message than sclera and choroid. We have mapped hCoch-5B2 to human 14q11.2–q13 by somatic cell hybrid analysis and FISH and, more precisely, using radiation hybrids to a region of markers linked to DFNA9, a nonsyndromic autosomal dominant sensorineural hearing loss with vestibular defects. Furthermore, we detect hCoch-5B2 on three overlapping YACs, two of which also contain one of the markers linked to DFNA9. mCoch-5B2 was genetically mapped in the mouse to chromosome 12, in a region of homologous synteny with human 14q11.2–q13, which contains the *asp1* (audiogenic seizure prone) locus in the mouse. © 1997 Academic Press

INTRODUCTION

Hearing loss is a heterogeneous disorder that affects over 14 million people in the United States, with approximately 1 of every 1000 infants being affected by congenital deafness. An estimated one-half of congenital hearing loss cases are due to genetic causes (Bieber and Nance, 1979). More than 175 different forms of hereditary deafness have been characterized, including autosomal dominant, autosomal recessive, X-linked, and mitochondrial forms (McKusick, 1994).

Genetic heterogeneity in hearing disorders both associated with other clinical anomalies (syndromic) and occurring as an isolated finding (nonsyndromic) indicates the involvement of a large number of genes in the complex development and function of the hearing process. Of the several hundred syndromic hearing loss disorders described (Gorlin *et al.*, 1995), only about 60 have been mapped to human chromosomes, with approximately half of these with characterized gene defects (reviewed by Duyk *et al.*, 1992; Petit, 1996). The majority of congenital hearing disorders are nonsyndromic (Cohen and Gorlin, 1995), but even fewer nonsyndromic disorders have been identified. This number is increasing through the study of consanguineous geographically isolated families. Over 30 human chromosomal loci associated with nonsyndromic hearing impairment have been identified, some with corresponding mouse mutants in the homologous region (reviewed by Petit, 1996; Van Camp *et al.*, 1997). However, to date, only three human nuclear genes responsible for nonsyndromic hearing impairment have been discovered: POU3F4 in DFN3 (de Kok *et al.*, 1995), MYO7A

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in DFNB2 (Liu *et al.*, 1997; Weil *et al.*, 1997), and GJB2 in DFNB1 and DFNA3 (Kelsell *et al.*, 1997).

To study the molecular biology of hearing and deafness, we have constructed a human fetal cochlear cDNA library. Identification of cochlear genes and their structure, expression pattern, and chromosomal location may help identify candidate genes for both syndromic and nonsyndromic hearing disorders. We previously reported identification of a partial cDNA for a highly expressed, novel human cochlear gene, hCoch-5B2,[3] isolated by subtractive hybridization and differential screening methods (Robertson *et al.*, 1994). Now, we report isolation of the highly conserved mCoch-5B2 mouse homolog and the complete deduced amino acid sequences, chromosomal locations, and expression patterns of both human and mouse Coch-5B2. Structural features of Coch-5B2, suggestive of a novel secreted protein, including homology to von Willebrand factor A domains present in a number of secreted proteins, such as extracellular matrix components, are discussed. Differences in size, number, level, and expression pattern of Coch-5B2 transcripts in human and mouse tissues are detected. Precise chromosomal localization of hCoch-5B2 shows overlap with the map location on human chromosome 14 of DFNA9, a nonsyndromic autosomal hearing disorder with vestibular defects (Manolis *et al.*, 1996). Further analysis of contiguous YACs from this region has been performed to assess colocalization of hCoch-5B2 with STS markers linked to DFNA9. Mapping of mCoch-5B2 in the mouse to a region of homologous synteny to the human chromosome assignment and to a region of a mouse mutant is described. Localization of human Coch-5B2 to the region of DFNA9 markers, as well as its high level of expression only in human cochlea and vestibule, the only affected organs in DFNA9, identifies hCoch-5B2 as a candidate for this disorder.

MATERIALS AND METHODS

Isolation of cDNAs

To obtain full length cDNA sequence of hCoch-5B2, we screened a human fetal brain cDNA library cloned in the Lambda Zap II vector (Stratagene, La Jolla, CA), using a hCoch-5B2 probe approximately 600 bp in size (ending at the first polyadenylation site), which we had originally isolated by subtractive hybridization and differential screening (Robertson *et al.*, 1994). Although hCoch-5B2 is expressed at a very low level in the brain, we chose this library because it is a mixture of oligo(dT)- and random-primed cDNAs and is more likely to contain 5′ ends of cDNAs.

Approximately 10^6 recombinant phage were screened using standard techniques. Filters were prehybridized at 42°C and then hybridized with ^{32}P-labeled random-primed (Feinberg and Vogelstein, 1984) 600-bp hCoch-5B2 probe and again with a cDNA probe approximately 580 bp from the most 5′ region of the cDNAs from the first screening, at 42°C for 48 h in 10% dextran sulfate, 4× SSC, 7 mM Tris–HCl (pH 7.6), 0.8× Denhardt's solution, 200 μg/ml sonicated herring sperm DNA, 40% formamide, and 0.5% SDS. Filters were washed in 0.1× SSC, 0.1% SDS at 50°C, prior to autoradiography using XAR-5 film (Eastman Kodak Co., Rochester, NY) and intensifying screens at −80°C. Following discovery of several cloning artifacts in this library, we then screened our original human fetal cochlear cDNA library. Filters were probed with a PCR-generated ^{32}P-labeled probe of approximately 230 bp using oligonucleotides GATTGTAAAGCAGACATTGC and ACCTACTTCCTTTATGGC from the most 5′ region of the available hCoch-5B2 cDNA from the previous screening. PCR was performed in 1× reaction buffer (Perkin–Elmer Cetus, Norwalk, CT); 0.8 μM each primer; 0.2 mM each dATP, dGTP, and dTTP; 0.01 mM dCTP; 100 μCi of [^{32}P]dCTP; and 1.25 units of *Taq* DNA polymerase. An initial denaturation was done at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 s, extension at 72°C for 1 min 30 s, and a final extension at 72°C for 7 min.

To obtain the 5′ end sequence of hCoch-5B2, we performed a final screening of our newly constructed human fetal cochlear CapFinder (Clontech, Palo Alto, CA) cDNA library (see below) using a probe generated from the same oligonucleotides and in the same manner as the previous screening.

To clone the mouse homolog of hCoch-5B2, a postnatal day 20 mouse oligo (dT)-primed brain cDNA library constructed in Uni-ZAP XR (Stratagene) was screened with the original 600-bp hCoch-5B2 ^{32}P-labeled random-primed cDNA probe in the manner described above.

Construction of Human Fetal Cochlear CapFinder cDNA Library

Total cellular RNAs were extracted (Chirgwin *et al.*, 1979) from cochlea (membranous labyrinths) obtained from human fetuses at 18–22 weeks developmental age, in accordance with guidelines established by the Human Research Committee at the Brigham and Women's Hospital. Small aliquots of these RNAs were run on denaturing agarose gels to assess RNA quality. Only samples without degradation were pooled for library construction. Poly(A)$^+$ RNAs were selected (Aviv and Leder, 1972) and used to construct a CapFinder (Clontech) long-distance PCR-based cDNA library, cloned into the Lambda Zap II vector (Stratagene). The oligo(dT) primer, high-fidelity, long-reading DNA polymerase, and a unique CapSwitch oligonucleotide are designed to select for 5′ cap of RNAs and to enrich for large, full-length cDNAs in this library.

Isolation of Genomic Clones

A human male placenta genomic library in Lambda FIX II (Stratagene) was screened in the same manner as the cDNA library, with a PCR-generated, ^{32}P-labeled hCoch-5B2 544-bp cDNA probe (ending at the first polyadenylation site) using oligonucleotides GGGCAGTCCTATGATGATGT and GCTATGGAATTTGCATATCT, for isolation of genomic clones to be used as probes for FISH mapping.

Nucleotide Sequence Analysis

Nucleotide sequence of clones was determined by the dideoxy chain termination method (Sanger *et al.*, 1977), using an ABI fluorescent DNA sequencing apparatus (Applied Biosystems, Foster City, CA). Sequence analysis was performed using the University of Wisconsin Genetics Computer Group software (Devereux *et al.*, 1984). Comparison of sequences to those deposited in nucleotide and peptide databases was performed using the BLAST Network Service of the National Center for Biotechnology Information (Altschul *et al.*, 1990).

Northern Blot Analysis

Total cellular RNAs were extracted (Chirgwin *et al.*, 1979) from second-trimester human fetal tissues, including membranous labyrinths (cochlea), vestibule, brain (cerebrum), spleen, and thymus and from human adult tissues, including brain (cerebrum), cerebellum, spinal cord, spleen, lymph node, lung, skeletal muscle, and skin. All human tissues were obtained in accordance with guidelines estab-

[3] The HGMW-approved symbol for the gene described in this paper is D14S564E.

lished by the Human Research Committee at Brigham and Women's Hospital. Human adult tissues were obtained from autopsies, except for the adult human spleen that was obtained from an individual with non-Hodgkin's lymphoma with splenomegaly, containing both normal and neoplastic cells. Total RNAs were extracted from normal mouse adult tissues including brain (cerebrum), cerebellum, spleen, thymus, heart, lung, liver, kidney, small intestine, large intestine, testis, cartilage, skeletal muscle, eye, retina, sclera, and choroid. Ten micrograms of each of the RNAs was electrophoresed in denaturing 1% agarose-formaldehyde gels and transferred to GeneScreen (DuPont, Wilmington, DE) filters (Thomas, 1980). After transfer, ethidium bromide-stained RNAs were visualized on filters to confirm integrity, concentration, and even transfer of RNAs. Filters were prehybridized for 2–4 h and hybridized overnight at 42°C, in the same solution as described above, with ^{32}P-labeled probes (Feinberg and Vogelstein, 1984) corresponding to the original 600-bp hCoch-5B2 cDNA, a composite of the full-length hCoch-5B2 cDNAs, hCoch-5B2 cDNA of approximately 750 bp from only the region beyond the first polyadenylation site, and the full-length mouse Coch-5B2 cDNA. Filters were washed in 0.1% SDS in 0.1× SSC at 42–55°C prior to autoradiography using XAR-5 film with intensifying screens at −80°C.

Gene Mapping

Somatic cell hybrid mapping of human Coch-5B2. DNAs from the NIGMS human/rodent somatic cell hybrid mapping panel 1 (Drwinga *et al.*, 1993), consisting of 18 hybrids retaining from 1 to 19 human chromosomes, were digested with *Eco*RI, electrophoresed in a 0.8% agarose gel, and transferred to Genescreen (DuPont) as described (Southern, 1975). The filter was hybridized with ^{32}P-labeled 600-bp original hCoch-5B2 cDNA probe and washed at 42°C as described above. The panel was scored for presence or absence of a human Coch-5B2 hybridizing band to determine concordance or discordance with the reported human chromosome in each hybrid.

Fluorescence In Situ hybridization (FISH) of hCoch-5B2. A hCoch-5B2 cDNA of approximately 1.6 kb (ending at the second polyadenylation site) and a human genomic clone of approximately 16–18 kb in length corresponding to hCoch-5B2 were used separately as probes for FISH. Probes were labeled with digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN) using dNTPs obtained from the same manufacturer and the DNase I/DNA polymerase I mixture from the BioNick Labeling System (Gibco BRL, Gaithersburg, MD). DNA was coprecipitated with 5 μg of Cot-1 DNA (Gibco BRL) and resuspended in 1× TE at 100 μg/ml.

Hybridization of metaphase chromosome preparations from peripheral blood lymphocytes obtained from normal human males was performed with the labeled hCoch-5B2 probe at a concentration of 7.5 μg/ml in Hybrisol VI as previously described (Ney *et al.*, 1993). Digoxigenin-labeled probe was detected using reagents supplied in the Oncor Kit (Oncor, Gaithersburg, MD) according to the manufacturer's recommendations. Metaphase chromosomes were counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Oncor). Map position of the labeled hCoch-5B2 probe was determined by visual inspection of the fluorescent signal on the DAPI-stained metaphase chromosomes. Hybridization was observed with a Zeiss Axiophot microscope and photographs were prepared using the CytoVision Imaging System (Applied Imaging, Pittsburgh, PA).

Mapping the murine homolog of hCoch-5B2. Segregation of Coch-5B2 was compared with that of marker loci previously typed in a panel of DNAs derived from progeny of matings between female (C57BL/6J × CAST/Ei)F1 hybrids and male C57BL/6J. The panel consists of 144 samples that have been characterized for more than 300 loci (Johnson *et al.*, 1994). Restriction fragment length variants between the parental strains were detected with the original hCoch-5B2 cDNA approximately 600 bp in size (Robertson *et al.*, 1994).

Genetic linkage of mCoch-5B2 was detected with markers on mouse chromosome 12. Gene order was determined by minimizing meiotic crossover events using the computer program Map Manager (Manly, 1993).

Identification of YACs containing hCoch-5B2 and markers linked to DFNA9. A series of overlapping YACs from a contig spanning the region of DFNA9 and of hCoch-5B2 were obtained from Research Genetics, Inc. (Huntsville, AL). These six YACs are 748-D-11, 888-C-6, 949-A-9, 925-C-2, 746-F-10, and 964-F-6. Primers corresponding to STS markers D14S49, D14S121, and D14S54, which have high lod scores for linkage to DFNA9 (Manolis *et al.*, 1996), were also obtained from Research Genetics. PCR was performed on the six YACs using primers from the DFNA9 markers as well as hCoch-5B2 primers CATCAGAGGCAGCATTTGTA and TTGTAACCAGAA-GGCAGC to assess localization.

RESULTS AND DISCUSSION

Analysis of Human and Mouse Sequences

The original hCoch-5B2 600-bp cDNA was obtained by subtractive hybridization and differential screening of our human fetal cochlear cDNA library (Robertson *et al.*, 1994). A subsequent number of screenings yielded a composite of several overlapping cDNAs, showing an alternate polyadenylation site approximately 480 bp downstream of the first polyadenylation site. The original hCoch-5B2 cDNA was also used to screen a mouse fetal brain cDNA library yielding two full-length clones with two different polyadenylation signals approximately 440 bp apart, consistent with transcript sizes detected on Northern blots (see Expression Pattern in Human and Mouse). The nucleotide sequences of the human and mouse Coch-5B2 have been deposited in the nucleotide databases under Accession Nos. AF006740 and AF006741, respectively.

Comparison of human and mouse Coch-5B2 reveals 89% identity in the nucleotide sequences in the regions of the open reading frame (ORF). This homology drops abruptly after the translation stop codon, indicating lack of conservation in the 3′ untranslated region of the gene. The hCoch-5B2 sequence shows an ORF of 550 amino acid residues; the mCoch-5B2 sequence shows an ORF of 552 amino acids (Fig. 1). There is only one gap of 2 amino acids between the human and mouse sequences, which appears at the very amino terminus, with the human sequence showing 4 leucine repeats as opposed to 6 in the mouse. The human and mouse sequences show a very high degree of conservation: 94% identity of amino acids and 96% similarity, taking into account conservative amino acid changes (Fig. 1).

The Coch-5B2 sequences of both human and mouse show a start methionine that conforms to the Kozak rules of translation initiation in eukaryotes (Kozak, 1987). Analysis of the most amino-terminal portion of both the human and the mouse Coch-5B2 reveals a potential signal peptide that fits von Heijne's rules for signal peptides and their potential cleavage sites (von Heijne, 1986). The lowest degree of amino acid conservation between the human and the mouse sequences is seen in this region (Fig. 1), which would be cleaved off in forming the mature protein. The predicted proteolytic cleavage site would be at residue 26 and 28 in

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  1 MSAAWIPALGLG  VCLLLLPGPAGSEGAAPIAITCFTRGLDIRKEKADV 48
     ...:    .    :          .   .  ::
  1 .PSSR....C..AWLL.....RF.RA...V..PV................ 50

 49 LCPGGCPLEEFSVYGNIVYASVSSICGAAVHRGVISNSGGPVRVYSLPGR 98
          .      :                      :.
 51 ......S......F......................GT............ 100

 99 ENYSSVDANGIQSQMLSRWSASFTVTKGKSSTQEATGQAVSTAHPPTGKR 148
                           .             .        .
101 .......................A.............R........S... 150

149 LKKTPEKKTGNKDCKADIAFLIDGSFNIGQRRFNLQKNFVGKVALMLGIG 198
151 .................................................. 200

199 TEGPHVGLVQASEHPKIEFYLKNFTSAKDVLFAIKEVGFRGGNSNTGKAL 248
201 .................................................. 250

249 KHTAQKFFTVDAGVRKGIPKVVVVFIDGWPSDDIEEAGIVAREFGVNVFI 298
             . .
251 .........A.T...................................... 300

299 VSVAKPIPEELGMVQDVTFVDKAVCRNNGFFSYHMPNWFGTTKYVKPLVQ 348
                     .
301 .................A................................ 350

349 KLCTHEQMMCSKTCYNSVNIAFLIDGSSSVGDSNFRLMLEFVSNIAKTFE 398
351 .................................................. 400

399 ISDIGAKIAAVQFTYDQRTEFSFTDYSTKENVLAVIRNIRYMSGGTATGD 448
                              .        :
401 ..........................N........LA............. 450

449 AISFTVRNVFGPIRESPNKNFLVIVTDGQSYDDVQGPAAAAHDAGITIFS 498
      .           :                   .
451 ..A...........D...................R............... 500

499 VGVAWAPLDDLKDMASKPKESHAFFTREFTGLEPIVSDVIRGICRDFLES 548
               :
501 ...........R...................................... 550

549 QQ* 550

551 ... 552
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FIG. 1. Deduced amino acid sequences of human (top row) (550 residues) and mouse (bottom row) (552 residues) Coch-5B2 show a very high degree of conservation: 94% identity of amino acids (indicated by a dot on the bottom row) and 96% amino acid similarity (indicated by double and single dots between the two rows). Only the amino acids that differ in the mouse from the human are indicated on the bottom row. The two sequences differ in length by 2 amino acids, with 4 leucine repeats in the amino terminus in the human as opposed to 6 leucines in the mouse. A potential signal peptide sequence (indicated by a horizontal line) conforming to von Heijne's rules (von Heijne, 1986) is present, showing a hydrophobic core including the stretch of leucines and a predicted cleavage site at glycine residue 26 in the human and 28 in the mouse. The lowest degree of amino acid conservation between the human and the mouse sequences is seen in the area of the predicted signal peptide which would be cleaved off in the mature protein. The translation stop codon is indicated by an asterisk (*).

the human and the mouse, respectively. A stretch of hydrophobic residues (four leucines in the human and six leucines in the mouse) is present in this potential signal peptide. This region is also the most hydrophobic portion of the whole sequence, according to analysis by the Kyte–Doolittle hydrophobicity plot (Kyte and Doolittle, 1982).

Consistent with the prediction of a secreted protein as indicated by the presence of a potential signal peptide in Coch-5B2 are the absence of a transmembrane region and the presence of two domains with homology to the von Willebrand factor (vWF) type A domain (Fig. 2). Type A domains are regions of approximately 200 amino acid residues in length, often present in multiple numbers (and sometimes in tandem) within a single protein. A superfamily of genes with type A-like domains or "modules" includes proteins, all with ligand-binding properties, involved in various functions such as hemostasis (vWF), the complement system (C2 and Factor B), the immune system (integrins such as LFA-1, Mac-1,VLA-1,VLA-2, p150, and 95), and the extracellular matrix (cartilage matrix protein and collagens type VI, VII, XII, and XIV) (reviewed in Colombatti and Paolo, 1991; Colombatti *et al.*, 1993). With the exception of integrins, which are transmembrane proteins, the only molecules known to date to contain type A-like domains are secreted proteins. Moreover, the highest number of type A-like domains known to date are found in the extracellular matrix (ECM), in proteins such as Col VIα3, which has 11 type A-like modules, which make up almost all of the protein (Bonaldo *et al.*, 1990).

Figure 2 shows the sequence similarity of the two vWF type A-like domains of hCoch-5B2 to each other and to one of the type A-like domains of the following: human collagen 12 α1 (COL12A1), VA module (Gerecke *et al.*, 1997); human cartilage matrix protein (CMP), A1 module (Jenkins *et al.*, 1990); and human vWF, A3 module (Mancuso *et al.*, 1991). Not shown in Fig. 2 are type A domains of a number of other genes that also have a high degree of homology to type A domains of Coch-5B2, such as collagens type V (Bonaldo *et al.*, 1989, 1990; Koller *et al.*, 1989), VII (Parente *et al.*, 1991; Christiano *et al.*, 1994), and XIV (Wälchli *et al.*, 1993). These collagens are all nonfibrillar collagens, types XII and XIV being defined as members of the FACIT (fibril-associated collagens with interrupted triple helices) family of collagens (Olsen *et al.*, 1995). All of these "collagens" are hybrid molecules that have relatively short collagenous domains consisting of Gly-X-Y repeats, in addition to larger noncollagenous domains, including the vWF type A modules. Coch-5B2 lacks any collagenous domain and is therefore not a collagen, unlike a novel inner ear-specific collagen found in fish (Davis *et al.*, 1995). Furthermore, Coch-5B2 also lacks "fibronectin type III" repeats, which are regions of homology to fibronectin with cell-binding properties present in some collagens that also have vWF type A domains (Colombatti *et al.*, 1993).

CMP (Agraves *et al.*, 1987; Jenkins *et al.*, 1990), a major component of the ECM of nonarticular cartilage, consisting of approximately 500 amino acid residues, shows some structural similarities to Coch-5B2. It possesses two vWF A domains separated by a small EGF-like region in the absence of a collagenous domain or fibronectin III repeats. Another similarity in structure between Coch-5B2 and CMP resides in the position of cysteine residues that are immediately adjacent to the amino and carboxy termini of each of the type A domains (Fig. 3). There are no cysteine residues within

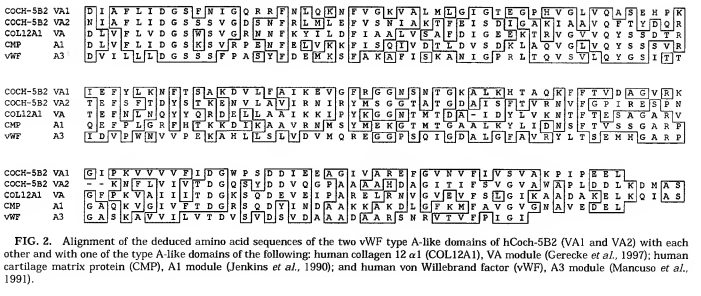

FIG. 2. Alignment of the deduced amino acid sequences of the two vWF type A-like domains of hCoch-5B2 (VA1 and VA2) with each other and with one of the type A-like domains of the following: human collagen 12 α1 (COL12A1), VA module (Gerecke *et al.*, 1997); human cartilage matrix protein (CMP), A1 module (Jenkins *et al.*, 1990); and human von Willebrand factor (vWF), A3 module (Mancuso *et al.*, 1991).

the type A domain itself. This same pattern of cysteines flanking but not within the type A domains is also seen in collagens type XII and XIV (Colombatti *et al.*, 1993). In CMP, it has been shown that the 2 cysteine residues flanking the A2 domain form an intramolecular disulfide bond, in addition to the intermolecular disulfide bonds formed between the cysteine residues outside the A domains for trimerization of CMP (Haudenschild *et al.*, 1995). Similar disulfide bonds may exist in Coch-5B2 between the 2 cysteine residues flanking the type A domains, in addition to a cluster of cysteines in the amino terminus of the sequence and 2 cysteines in the short intervening sequence between the two type A domains (Fig. 3).

Type A domains of various proteins are thought to mediate a variety of interactions between components of the ECM, cell–ECM interactions, cell–cell adhesion, and cell membrane receptor and soluble factor interactions via binding of the type A domain to proteins such as fibrillar collagens, hyaluronic acid, glycoprotein GpIb, heparin, and complement fragment iC3B, as reviewed by Colombatti *et al.*, (1993). The type A domains of von Willebrand factor have been shown to bind to fibrillar collagens types I and III (Roth *et al.*, 1986; Kalafatis *et al.*, 1987; Pareti *et al.*, 1987). Other collagens, types VI, XII, and XIV, and CMP are also thought to bind fibrillar collagens as a bridging role in ECM assembly and stabilization (Colombatti *et al.*, 1993). Interestingly, we have previously shown that the cochlea expresses very high levels of COL1A2 and COL3A1 (levels comparable to Coch-5B2) (Robertson *et al.*, 1994). It is possible that Coch-5B2 may interact via type A domains with the abundant fibrillar collagens for ECM assembly in the cochlea, where function is so tightly dependent on the highly structured architecture of this sensory organ.

Expression Pattern in Human and Mouse

We previously detected very high levels of hCoch-5B2 mRNA only in human fetal cochlea and very low levels in human fetal brain and eye, among a large panel of fetal tissues tested (Robertson *et al.*, 1994). We now have analyzed human adult tissues including brain, cerebellum, spinal cord, spleen, lymph node, lung, skeletal muscle, and spleen. (Adult human cochlea was not available.) High levels of hCoch-5B2 mRNA were not detected in any of the adult human tissues tested (data not shown), findings consistent with those in fetal tissues. Low levels of hCoch-5B2 message were detected in human adult muscle.

In addition, we have looked at hCoch-5B2 expression in human fetal vestibule. Very high level of expression of hCoch-5B2 is seen in the vestibule, comparable only to the level in the cochlea (data not shown). This finding is interesting in that these two organs have developmental, anatomical, and functional similarities and that cochlear and vestibular dysfunction may be found together frequently both in mice and in humans. In particular, a human deafness disorder, DFNA9 (Manolis *et al.*, 1996), for which hCoch-5B2 is a positional candidate (also discussed under Mapping in Human),

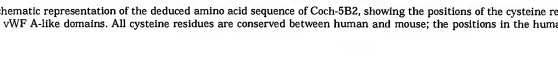

FIG. 3. Schematic representation of the deduced amino acid sequence of Coch-5B2, showing the positions of the cysteine residues with respect to the vWF A-like domains. All cysteine residues are conserved between human and mouse; the positions in the human sequence are indicated.

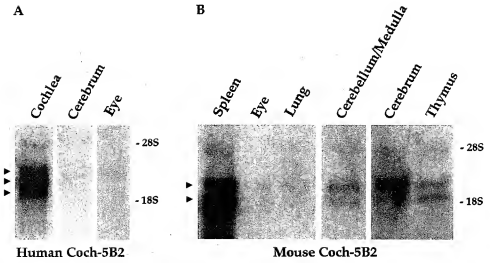

FIG. 4. (A) Autoradiograph of Northern blot of 10 μg of total RNAs extracted from human fetal cochlea, cerebrum (brain), and eye hybridized with a 600-bp hCoch-5B2 probe. Negative tissues (not shown) are human fetal spleen, liver, kidney, lung, skin, thymus, adrenal, small intestine, sternal cartilage, and cultured fibroblasts (Robertson *et al.*, 1994). Very high level of expression of hCoch-5B2 is seen in human fetal cochlea; very low levels are detected in cerebrum and eye. hCoch-5B2 transcripts are estimated as 2.0, 2.3, and 2.9 kb. (B) Autoradiograph of Northern blot of 10 μg of total RNAs extracted from mouse adult spleen, eye, lung, cerebellum/medulla, and cerebrum (brain) and hybridized with a 2.5-kb mouse Coch-5B2 cDNA probe. Negative tissues (not shown) are mouse adult heart, liver, small intestine, large intestine, kidney, sternal cartilage, skeletal muscle, and testis. Two transcripts approximately 2.0 and 2.5 kb in size were detected. Message sizes are in agreement with the isolated mouse cDNAs, which show two different polyadenylation sites approximately 440 bp apart. A high level of message is seen in the mouse spleen, lower levels are seen in the cerebrum, cerebellum/medulla, and thymus, and faint bands are detectable in eye and lung. mCoch-5B2 expression pattern in the mouse is somewhat different from a more specific expression pattern in the human. Transcripts are indicated by arrowheads; positions of 28S and 18S rRNAs are marked by lines.

has associated vestibular findings. Temporal bone sections from individuals affected with DFNA9 show accumulation of acidophilic deposits obstructing the cochlear and vestibular nerve channels, causing severe degeneration of dendrites and atrophy of cochlear and vestibular sense organs (Khetarpal *et al.*, 1991; Khetarpal, 1993).

Northern blot analysis of a panel of adult mouse tissues (Fig. 4B) reveals messages of approximately 2.0 and 2.5 kb, consistent in size with the two mouse cDNAs, with the two polyadenylation sites in the mouse, and approximately with two of three human messages (Fig. 4A). (The larger band in the mouse is approximately 2.5 kb, migrating slightly higher than the predominant human message of approximately 2.3 kb.) Three messages approximately 2.0, 2.3, and 2.9 kb in size are seen in the human (Fig. 4B). Northern analysis using a probe derived from the 3′ portion of hCoch-5B2 cDNA, excluding any sequence 5′ of the first polyadenylation site, shows hybridization only to the largest of the three hCoch-5B2 transcripts in the cochlea (data not shown), indicating that the second polyadenylation site is responsible for the largest hCoch-5B2 mRNA. The majority of our isolated cDNAs (8 of 10) possesses the first polyadenylation site, corresponding most likely to the middle-sized and the most predominant (highest level) of the three hCoch-5B2 transcripts. The smallest hCoch-5B2 mRNA may also be the result of usage of a different (more 5′) polyadenylation site (although not yet seen in any of our isolated clones) or may be a product of alternative splicing, exon skipping, differential use of promoter cap sites, or a different related gene. Alternative splicing has been reported in the vWF A-like domains of collagen VI α 3 (Doliana *et al.*, 1990).

The expression pattern in adult mouse differs from that in fetal and adult human (Fig. 4). Coch-5B2 is expressed abundantly in adult mouse spleen, at moderate levels in cerebrum, cerebellum/medulla, and thymus, and at low levels in eye and lung (Fig. 4B). High and moderate levels of mCoch-5B2 mRNA are seen in a wider variety of mouse tissues in contrast to a more specific cochlear and vestibular expression in the human. Notably, the mouse spleen expresses Coch-5B2 at a high level, whereas no expression is detectable in human fetal and adult spleen. This difference remains to be elucidated but may reflect the function of the spleen in hematopoiesis in the mouse compared to the human. Different expression patterns of Coch-5B2 in human and mouse may indicate some difference in the function of this gene in the two species and/or may be responsible for different disease phenotypes in the same mutated gene in the two species.

In both species, very low level Coch-5B2 expression is detected in the eye (Fig. 4). Expression of Coch-5B2 in the eye is of particular interest due to the finding of numerous disorders that affect both the auditory and the visual systems, such as the heterogeneous Usher syndrome (Fishman *et al.*, 1983). We have looked at the sensorineural portion vs the connective tissue and

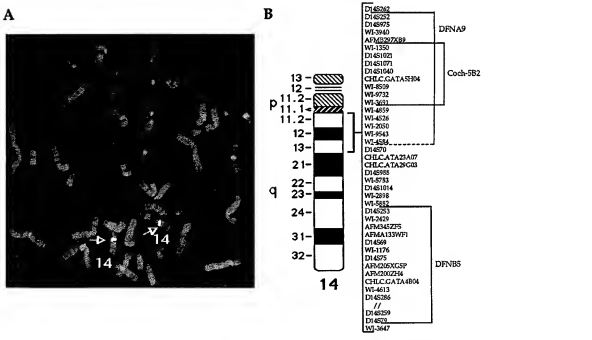

FIG. 5. Chromosome localization of the hCoch-5B2 gene. **(A)** Photograph of human metaphase chromosomes counterstained with DAPI following FISH with a hCoch-5B2 genomic probe. The two chromosomes 14 are indicated by numbers. Arrows point to the site of hybridization of the digoxigenin-labeled hCoch-5B2 probe on both chromosomes 14 in bands q11.2–q13. **(B)** Idiogram of human chromosome 14 with STS markers from the Whitehead Institute for Biomedical Research/MIT Center for Genome Research (http://www-genome.wi.mit.edu/) showing the map interval for hCoch-5B2 in relation to the map intervals of markers linked to two nonsyndromic deafness disorders, DFNB5 (Fukushima *et al.*, 1995) and DFNA9 (Manolis *et al.*, 1996). Human Coch-5B2 maps within the DFNA9 interval but does not overlap with the more telomeric DFNB5 markers. Markers linked to DFNA9 have been assigned positions on chromosome 14 by the Cooperative Human Linkage Center (http://www.chlc.org) and have not all been placed on the Whitehead/MIT map. The dashed line indicates that the most telomeric marker for DFNA9 is proximal to D14S70. A gap between two mapping contigs, spanning an interval of approximately 6 cM, is indicated by //.

supportive portion of the eye by Northern analysis: expression of mCoch-5B2 is high in mouse retina and very low to undetectable in sclera and choroid (data not shown). This preliminary finding can be explored further in terms of relevance in a sensorineural system. Although we did not have mouse cochlear RNA for these studies, the mouse homolog of Coch-5B2 has been isolated from a mouse inner ear cDNA library (GenBank Accession No. Z78163), supporting its expression and likely conserved and fundamental role in inner ear biology. Our mouse clone (isolated from a mouse brain cDNA library) matches the sequence that was obtained from the mouse inner ear cDNA library.

Mapping in Human

Physical mapping of hCoch-5B2 may point toward a region of the human genome to which human deafness disorders have been mapped and may provide a positional candidate for the disorder. We localized hCoch-5B2 to human chromosome 14 by Southern blot of the NIGMS human/rodent somatic cell hybrid panel 1 (Drwinga *et al.*, 1993) probed with a 600-bp hCoch-5B2 cDNA (data not shown). A human-specific hybridizing band of approximately 5.7 kb in size was detected. Hybridizing bands to mouse and hamster genomic DNA, approximately 8.1 and 3.3 kb, respectively, were also detected, showing the evolutionary conservation of this gene. Map assignment was done on the basis of lowest percentage of discordancy (6%) of segregation of the hCoch-5B2 hybridizing band with human chromosome 14 in 18 somatic cell hybrids. The hybrid cell line in which a hCoch-5B2-hybridizing band could not be detected shows only 2% of the cells examined to have retained chromosome 14. It is possible that this level was below the sensitivity of detection of our Southern blot.

For a more precise map assignment, we performed fluorescence *in situ* hybridization. Map position was determined by visual inspection of the fluorescent hybridization signals on DAPI-stained metaphase chromosomes. When a human Coch-5B2 cDNA probe of approximately 1.6 kb was used, signal was detected on the long arm of chromosome 14 in band q11.2–q13 (Fig. 5A) in 20 metaphase preparations. In 3 metaphases, signal was detected on both chromosomes 14. This map assignment was confirmed by performing FISH using a hCoch-5B2 genomic clone of approximately 16–18 kb in size. In 10 of 12 metaphase preparations analyzed, hybridization signal was present on q11.2–q13 (Fig. 5A), in 9 metaphase spreads, both copies of chromosome 14 were labeled, and in 1 metaphase spread, sig-

TABLE 1

Presence of Coch-5B2 and DFNA9 Markers on YACs

YACs	Primer Pairs			
	Coch-5B2	D14S54	D14S121	D14S49
784-D-11	–	–	–	–
888-C-6	+	–	–	–
949-A-9	+	+	–	–
925-C-2	+	+	–	–
746-F-10	–	+	–	–
964-F-6	–	–	+	+

Note. Summary of presence (+) or absence (–) of PCR product using primer pairs from hCoch-5B2 and three STS markers, D14S54, D14S121, and D14S49, with high lod scores for linkage to DFNA9 (Manolis *et al.,* 1996) on a series of overlapping YACs from a contig on human chromosome 14 spanning the region of DFNA9-linked markers and of hCoch-5B2 localization. hCoch-5B2 was present on three overlapping YACs (888-C-6, 949-A-9, and 925-C-2), two of which also contained one of the STS markers (D14S54) linked to DFNA9. The three STSs have not been placed on the Whitehead/MIT map (Fig. 5), but have been place on a Cooperative Human Linkage Center map of human chromosome 14 within the interval outline for DFNA9 in Fig. 5.

nal was detected on one chromosome 14. (The official Human Gene Mapping nomenclature for hCoch-5B2 is D14S564E, GDB Accession No. G00-335-416.)

Of particular interest are two nonsyndromic deafness disorders that have been mapped to this region of the proximal long arm of chromosome 14 by linkage analysis in two kindreds. One is DFNB5, a nonsyndromic autosomal recessive hearing loss, which has been localized to an approximately 9-cM region defined by markers D14S253 and D14S79, which are linked to DFNB5 (Fukushima *et al.,* 1995) (Fig. 5B). The other disorder is DFNA9, a nonsyndromic autosomal dominant sensorineural hearing loss with vestibular defects (Khetarpal *et al.,* 1991; Khetarpal, 1993; Manolis *et al.,* 1996) (also discussed under Expression Pattern in Human and Mouse). Pedigree analysis of the DFNA9 kindred suggests that the mutant gene has complete penetrance. Hearing loss begins in the third decade and is variably progressive with high frequencies affected first, followed by middle and low frequencies. DFNA9 maps within a 9-cM interval from D14S252 to D14S49 (Fig. 5B), which is centromeric to the DFNB5 map assignment.

To evaluate where hCoch-5B2 maps with respect to these two disease loci and in relation to markers on the Whitehead map, a more precise map assignment was needed. We performed a BLAST search of dbSTS, which resulted in the identification of an STS identical to hCoch-5B2 sequence designated WI-12411, which maps by radiation hybrid analysis to 14q12–q13 (Fig. 5B), in agreement with our FISH assignment. hCoch-5B2 maps to an interval that does not overlap with the DFNB5 locus (Fig. 5B) and is therefore excluded as a positional candidate for this disorder. However, the map position assigned to WI-12411 by the Whitehead Institute/MIT Center for Genome Research is between markers AFMB297XB9 and WI-4859, placing hCoch-5B2 completely within the interval for DFNA9, indicating that hCoch-5B2 is a candidate for this disorder.

To assess further the map position of hCoch-5B2 in relation to the STS markers that are linked to DFNA9, we have evaluated the presence of hCoch-5B2 and DFNA9 disease markers on a series of overlapping YACs spanning the disease locus. Primer pairs from three STSs (D14S54, D14S121, and D14S49) with highest lod scores for linkage to DFNA9 (Manolis *et al.,* 1996) were used for PCR on six YACs (784-D-11, 888-C-6, 949-A-9, 925-C-2, 746-F-10, and 964-F-6). Primers from hCoch-5B2 were also used to detect presence of this gene on these YACs. Table 1 summarizes the presence (+) or absence (–) of the hCoch-5B2 gene on the designated YAC. hCoch-5B2 was present on three overlapping YACs, two of which also contained one of the STS markers linked to DFNA9. These data further confirm hCoch-5B2 as a strong positional candidate gene for DFNA9, warranting mutation analysis of this gene for this disorder.

Mapping in Mouse

Mapping of mCoch-5B2 may suggest a disease locus in the mouse and may provide an animal model for the study of this gene. Many mouse mutants with hearing disorders have been identified, some of which have known affected genes or loci (reviewed by Steele and Brown, 1994). Interestingly, most of these mouse mutants have associated vestibular defects as indicated by phenotypes and behaviors for which they were named, such as shaker, spinner, and waltzer (reviewed by Petit, 1996).

We have mapped mCoch-5B2 to chromosome 12 (Fig. 6), in a region of homologous synteny to human

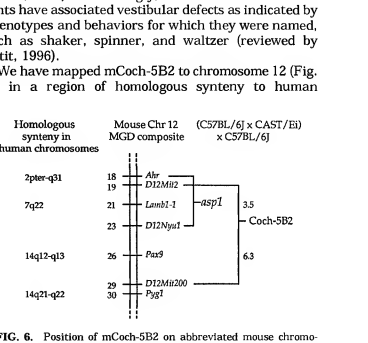

FIG. 6. Position of mCoch-5B2 on abbreviated mouse chromosome 12. A genetic map was constructed from the (C57BL/6J × CAST/Ei) × C57BL/6J backcross; interlocus recombination distances (3.5 and 6.3 cM) are indicated. A composite map derived from the MGD with mouse locus position assignments (approximate distances in centimorgans from the centromere) is shown. Homologous human chromosome assignments are given on the left of the chromosome. The position for *asp1* (audiogenic seizure prone) between *Ahr* and *D12Nyu1* (Neumann and Seyfried, 1990) is indicated.

14q11.2–q13. Segregation of mCoch-5B2 was compared with that of marker loci previously typed in a panel of DNAs derived from progeny of matings between female (C57BL/6J X CAST/Ei)F1 hybrids and male C57BL/6J. Restriction fragment length variants of 9.0 kb in C57BL/6J and 5.9 kb in CAST/Ei were detected in a *TaqI* digest probed with the 600-bp hCoch-5B2 cDNA. Gene order was determined on chromosome 12, placing mCoch-5B2 between the markers D12Mit2 and D12Mit200 (Fig. 6), which have been located at positions 19 and 29, respectively, on the Mouse Genome Database (MGD) composite map of chromosome 12 (Mouse Genome Database, 1997). (The mouse mapping data have been deposited with the MGD under Accession No. MGD-JNUM-40510. The official Mouse Gene Mapping nomenclature for mCoch-5B2 is D12H14S564E.) The human and mouse map assignments are consistent with previously defined human–mouse conserved gene arrangements between mouse chromosome 12 and human chromosome 14. For example, *Pax9* has been placed at position 26 on mouse 12 (Mouse Genome Database, 1997) near where we have localized mCoch-5B2. The human homolog of *Pax9* maps to the q11–q13 region of human chromosome 14.

This region on mouse 12 to which mCoch-5B2 maps contains the *asp1* (audiogenic seizure prone) locus characterized by susceptibility to sound-induced convulsions (Collins and Fuller, 1968). Using BXD recombinant inbred strain analysis, *asp1* was mapped between *Ahr* and *D12Nyu1* on mouse chromosome 12 (Neumann and Seyfried, 1990), corresponding to a position between 18 and 23 on the MGD composite map (Fig. 6).

Although *asp1* in the mouse and DFNA9 in the human are phenotypically very different, it is not known whether they could represent involvement of homologous genes in the two species. By chromosomal localization, Coch-5B2 can be considered a candidate for DFNA9 and/or *asp1*. Further colocalization of hCoch-5B2 and DFNA9 markers to the same YACs, as well as high level expression of hCoch-5B2 only in the cochlea and vestibule (the two affected organ systems in DFNA9), further warrant the pursuit of hCoch-5B2 as a strong candidate for the nonsyndromic DFNA9.

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